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THE MEASUREMENT OF METHEMOGLOBIN*

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Methemoglobin is formed when iron of the hemoglobin molecule is changed from the bivalent ferrous form to the trivalent ferric form, which does not combine further with nor yield oxygen. This conversion of hemoglobin to methemoglobin occurs when certain amino and nitro compounds of the aromatic series are absorbed through the skin, inhaled, or ingested. The compounds forming methemoglobin include such therapeutic drugs as acetanilid, phenacetin, sulfonamides, and nitrites, and such industrial and analytical chemicals as aniline, nitrobenzene, dinitrobenzene, and some of their derivatives.

In cases of methemoglobinemia, the patients exhibit a dusky blue cyanosis and samples of their blood have a chocolate brown color. The severity of the cyanosis is believed to be directly proportional to the degree of methemoglobinemia.[6] Therefore, accurate measurement of the methemoglobin in the blood is valuable not only to confirm the diagnosis, but also to evaluate results of treatment of methemoglobinemia.

Spectrophotometric determinations of mixtures of biological pigments began in 1876 with the work of Vierodt.[4] He originated a method for measuring the amounts of two pigments in a common solvent. His method was unique in that he measured the absorption ratios of the two components of the single solution at two different wave lengths. In 1900 Hufner[7] modified this procedure and succeeded in measuring the proportions of reduced hemoglobin and oxyhemoglobin, and of carboxyhemoglobin and oxyhemoglobin, in mixed solutions. In 1933 Heilmeyer[7] published his excellent monograph on quantitating mixed solutions of blood pigments, including mixtures of methemoglobin and oxyhemoglobin.

Up to this time all transmission measurements had been made with visual spectrometers. These were instruments of excellent precision, but one needed considerable skill and experience to operate them and to interpret the results. The perfection of photoelectric cells in the 1930's provided the means to expand the scope of this work.

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In 1935 Drabkin and Austin[1,3,4] worked out a method and the formulae for the determinations of methemoglobin and oxyhemoglobin. Their procedure was laborious, time consuming, and required large blood samples. These disadvantages limited the use of this method for clinical work. In 1938 Hamblin and Mangelsdorff[6] developed a method to measure the component percentages of methemoglobin and oxyhemoglobin in a single solution. They used a General Electric automatic recording spectrophotometer. The method proved to be rapid and accurate. The calculation of component percentages was done by geometrical alignment with standard transmission curves, which compensated for variations in sample oncentration. However, use of this method was restricted to those laboratories which were fortunate in having such an instrument.

During the same year Evelyn and Malloy[5] reported a method to determine oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single blood sample using a photoelectric photometer. Difficulties reported with this method included turbidity of the solutions, use of a highly colored reagent with glass filters having wide wave bands, and the conversion charts obtained on semi-log paper were usually curves rather than straight lines. In 1944 Horecker and Brackett[8,9] published a method for the simultaneous determination of methemoglobin, carboxyhemoglobin, and oxyhemoglobin by measuring transmission at isobestic wave lengths and at wave lengths in the infrared. However, this method also posed the problem of an expensive and uncommonly available instrument.

It was desirable to develop a method or adapt an existing one for more general use. Such a procedure should require:

1. Quantitation in the visible spectrum (400 to 700 mμ), which is available on the usual clinical laboratory spectrophotometer or photometer.

2. A reasonably simple and rapid technique.

3. A calculation, which compensates for differences in total hemoglobin concentration, to give the precision and accuracy needed.

We decided to try to modify or to adapt the method of Hamblin and Mangelsdorff[6] for use on a clinical spectrophotometer. Two reasons influenced this decision. First, their method had proved to be very accurate, without being tedious and time consuming. Second, we were fortunate to have available an automatic recording spectrophotometer so that replicates could be done to verify the results on the clinical instrument. Our first work was done with a Coleman #11 Universal Spectrophotometer, which is currently in use in our laboratory. In addition the procedure has been set up successfully on a Leitz "Photometer" and on a Bausch and Lomb "Spectronic 20." It would appear that this procedure could be used satisfactorily on similar clinical laboratory instruments. Further, it is likely that analogous procedures could be developed for measuring other pigments in addition to methemoglobin and oxyhemoglobin.

PRINCIPLES

If one can safely assume the absence of other hemoglobin pigments, it follows that the concentration of oxyhemoglobin plus the concentra-

tion of methemoglobin will equal the concentration of the total hemoglobin. It is then possible to measure both components in a single aqueous solution,[2] and so reduce the technical errors of sampling and dilution. The degree of dilution will depend on the sensitivity of the photo-cell, the amplification of the galvanometer current, and the strength of light energy in the instrument used.

The percent concentrations of oxyhemoglobin (neutral) and methemoglobin (neutral) in a dilute, aqueous solution are mathematically related to the transmittance of that solution.[2] To find this relation transmittance is measured at a total isobestic wave length and then at a wave length of wide separation. A total isobestic wave length is that wave length at which the transmission values are identical for all components of a mixture. In contrast, a wave length of wide separation is one at which the transmission values for equal concentrations of these components show a maximum difference. The calculation of the component percentage may be made from simple, first-order, algebraic equations.

METHOD

Procedure for Unknown Determinations

Measure 10 ml. of neutral distilled (or deionized) water into each of two Pyrex centrifuge tubes (15 ml. capacity) for each determination. Withdraw 1 to 2 ml. of venous blood with a clean, dry syringe. Avoid stasis of blood in the vein and excess pressure on the syringe plunger. Transfer the blood to a small, clean, dry test tube. Before coagulation starts add 0.1 ml. of blood to each tube of water, which was previously prepared. Avoid aeration of the blood or diluting fluid in these manipulations.

Stopper and invert the tubes gently. Allow the dilutions to stand for at least two to three minutes for complete hemolysis. Then centrifuge the tubes for ten minutes at approximately 2,000 r.p.m. Pour off the supernatant fluid at once. Do not disturb the sediment, for the fluid must be crystal clear.[10]

Immediately read the optical densities (or %T and convert) at 520 mμ and at 620 mμ. Compute the % methemoglobin from the standard formula, which as shown in the derivation and calculation, is

$$\% \text{ methemoglobin} = 100 \left[A - B \left(\frac{D_{620}}{D_{520}} \right) \right].$$

Procedure for Standardization

Select several individuals (10 to 12), who can be assumed to be free of methemoglobinemia. Prepare centrifuged dilutions of fresh, whole blood from each of these individuals. The dilutions and procedures should be identical with those used in unknown determinations. Read the supernatant fluid at 520 mμ and at 620 mμ. Record in terms of optical density. These readings, of course, correspond to 100% oxyhemoglobin or 0% methemoglobin.

Return the supernatant fluid to the same centrifuge tubes. To convert all the hemoglobin to methemoglobin,[4,6] add to each tube 0.1 ml. of fresh 10% solution of potassium cyanide (C.P., Analytic Reagent Grade). Stopper and invert the tubes gently. Allow to stand at least three minutes for complete conversion.

Recentrifuge and again read the clear supernatant fluid at 520 mμ and at 620 mμ. Record in terms of optical density. Similarly, these readings correspond to 100% methemoglobin.

Since both readings at 520 mμ are made at a total isobestic point and the concentrations of the components are equal, the density readings must be identical, within the limits of instrumental error.

Derivation of the Formula

To derive the formula, establishing the relation between the component concentrations and the densities, we must revert to the fundamental formula of the Bouguer-Beer Law, which is $C = D/K$ or $D = CK$. Moreover, at a given wave length, the total density of a mixed solution equals the sum of the densities of the components of the mixture, or $D = D_1 + D_2$.

Therefore, $D = C_1K_1 + C_2K_2$. (Eq. 1)

To apply these general formulae in the problems which must be solved, substitution of a more specific notation is necessary.

Let x = the concentration of oxyhemoglobin
y = the concentration of methemoglobin
S = the concentration of total pigment
D_{520} = observed density at 520 mμ
D_{620} = observed density at 620 mμ
k_{x520} and k_{y520} = K_1 and K_2 factors respectively, at 520 mμ
k_{x620} and k_{y620} = K_1 and K_2 factors respectively, at 620 mμ

Since $x + y = S$, it is obvious that

$$x = S - y, \quad \text{(Eq. 2)}$$

and

$$y = S - x. \quad \text{(Eq. 3)}$$

Substituting the above values in Eq. 1,

$$D_{620} = k_{x620}x + k_{y620}y. \quad \text{(Eq. 4)}$$

Then, from Eq. 2,

$$D_{620} = k_{x620}x + k_{y620}S - k_{y620}x, \quad \text{(Eq. 4a)}$$

and from Eq. 3,

$$D_{620} = k_{x620}S - k_{x620}y + k_{y620}y. \quad \text{(Eq. 4b)}$$

However, at the isobestic point (520 mμ), $k_{x520} = k_{y520}$.
So, Eq. 4a simplifies to

$$D_{620} = k_{y620}S, \quad \text{(Eq. 5)}$$

and Eq. 4b simplifies to

$$D_{620} = k_{x620}S. \quad \text{(Eq. 6)}$$

Obviously, Eq. 5 and Eq. 6 are identical.

At the point of maximum difference in transmission (620 mμ), $k_{x620} \neq k_{y620}$, so that similarly substituting in Eq. 1,

$$D_{620} = k_{x620}x + k_{y620}S - k_{y620}x, \qquad \text{(Eq. 7)}$$

and

$$D_{620} = k_{x620}S - k_{x620}y + k_{y620}y \qquad \text{(Eq. 8)}$$

Then, solving for x in Eq. 7 and y in Eq. 8

$$x = \frac{D_{620} - Sk_{y620}}{k_{x620} - k_{y620}}, \qquad \text{(Eq. 9)}$$

and

$$y = \frac{Sk_{x620} - D_{620}}{k_{x620} - k_{y620}} \qquad \text{(Eq. 10)}$$

To find the percent of each component, multiply Eq. 9 and Eq. 10 by 100/S, and simplify.
Therefore,

$$\% x = 100\left[\frac{D_{620}}{S(k_{x620} - k_{y620})} - \frac{k_{y620}}{k_{x620} - k_{y620}}\right], \qquad \text{(Eq. 11)}$$

and

$$\% y = 100\left[\frac{k_{x620}}{k_{x620} - k_{y620}} - \frac{D_{620}}{S(k_{x620} - k_{y620})}\right]. \qquad \text{(Eq. 12)}$$

However, from Eq. 5, $S = \frac{D_{520}}{k_{y520}}$; and if this value of S is substituted in Eq. 12,

$$\% y = \left[\frac{k_{x620}}{(k_{x620} - k_{y620})} - \left(\frac{D_{620}}{D_{520}}\right)\left(\frac{k_{y520}}{k_{x620} - k_{y620}}\right)\right] \qquad \text{(Eq. 14)}$$

If we let $A = \frac{k_{x620}}{k_{x620} - k_{y620}}$, and $B = \frac{k_{y520}}{k_{x620} - k_{y620}}$,
and substitute these values in Eq. 14, then

$$\% \text{ methemoglobin} = 100\left[A - B\left(\frac{D_{620}}{D_{520}}\right)\right]$$

This equation establishes the linear relation between the ratio of the densities of the solution and the relative concentration of methemoglobin.

Calculation

To apply the formula, first determine the ratios of the observed densities, for the samples containing 0% and 100% methemoglobin as measured in the procedure for standardization. Then compute the average ratio for both concentrations of methemoglobin. (If the density ratio for 0% methemoglobin of a sample is obviously "out of line", the sample probably contained an abnormal hemoglobin pigment and should be discarded in finding the average ratios.) Substituting the average ratios and the corresponding percentages of methemoglobin in the formula, gives two simultaneous equations. The solution of these equations will determine the values of A and B for the instrument. These particular A and B values are then used in the formula, to solve for unknowns. Substitution of the observed densities of the unknown dilution will determine the unknown % methemoglobin.

DISCUSSION

Results of an actual standardization procedure are shown in Table I. In this experiment, the average ratios of the densities are 0.085 for 0% methemoglobin and 0.521 for 100% methemoglobin. Substituting these

values in the standard formula, 0% methemoglobin = 100 (A — 0.085B) and 100% methemoglobin = 100 (A — 0.521 B). Solving the two simple simultaneous equations, A = —0.194905 and B = —2.293. Thus, the standard formula for this particular instrument becomes:

$$\% \text{ methemoglobin} = 100\left[\ (-0.194905) - (-2\text{-}293)\left(\frac{D_{650}}{D_{520}}\right)\right]$$

This can, of course, be simplified to:

$$\% \text{ methemoglobin} = (229.3)\left(\frac{D_{650}}{D_{520}}\right) - 19.4905$$

TABLE I

Specimen No.	0% METHEMOGLOBIN			100% METHEMOGLOBIN		
	D_{520}	D_{650}	D_{650}/D_{520}	D_{520}	D_{650}	D_{650}/D_{520}
1	0.854	0.071	0.083	0.854	0.444	0.520
	0.854	0.071	0.083	0.854	0.444	0.520
2	0.921	0.079	0.086	0.921	0.482	0.523
	0.903	0.079	0.087	0.903	0.469	0.519
3	1.000	0.088	0.088	1.000	0.523	0.523
	0.979	0.086	0.088	0.979	0.509	0.520
4	0.959	0.084	0.088	0.959	0.502	0.524
	0.959	0.081	0.084	0.959	0.502	0.524
5	1.046	0.089	0.085	1.046	0.546	0.522
	1.046	0.089	0.085	1.046	0.541	0.522
6	0.886	0.074	0.084	0.903	0.469	0.518
	0.903	0.076	0.084	0.903	0.469	0.518
7	0.903	0.076	0.084	0.903	0.469	0.518
	0.886	0.076	0.086	0.903	0.469	0.518
8	1.000	0.084	0.084	1.000	0.523	0.523
	1.000	0.086	0.086	1.000	0.523	0.523
9	0.939	0.081	0.086	0.939	0.489	0.521
	0.939	0.081	0.086	0.939	0.489	0.521
10	0.939	0.079	0.084	0.939	0.489	0.521
	0.939	0.079	0.084	0.939	0.489	0.521
11	0.921	0.076	0.082	0.921	0.482	0.522
	0.903	0.074	0.082	0.921	0.482	0.522
12	0.886	0.076	0.086	0.886	0.462	0.521
	0.886	0.076	0.086	0.903	0.469	0.519
Average	....	....	0.085	....	....	0.521
Range	....	....	0.082–0.088	....	....	0.518–0.524

When these constants have been determined, the only computation actually necessary is the division of the densities to find their ratio. The standard formula is a first-order algebraic equation, which will plot a straight line on rectilinear coordinate paper with values of the density ratio on the abscissa and the % methemoglobin on the ordinate. A more sophisticated means of determining the ratios of the densities involves the construction of a nomogram. The efficiency gained is well worth the effort required to make one, since the use of the nomogram and the graph obviates the need for any actual computation.

In our own work we have had occasion to perform this determination within a rather limited range of total hemoglobin values. All of our personnel undergo pre-employment and annual examinations, which include a hemoglobin determination by Drabkin's cyanmethemoglobin method, a Wintrobe hematocrit, a careful study of a stained blood smear, and if indicated, a red blood cell count. If we found an individual's hemoglobin concentration or hematologic indices were not well within acceptable normal values, he would be required to avoid contact with cyanosis-producing materials. Nevertheless, it is important to establish the total hemoglobin range, through which the method is accurate. In order to determine the effectiveness of compensating for differences in total hemoglobin, a variety of volumetric dilutions were prepared from a single blood sample. In these dilutions the amount of blood was held constant, while the volume of the solvent was changed. 10.1 ml. portions of each dilution were used in the procedure for standardization. The original blood specimen contained 14.0 g. total hemoglobin per 100 ml., determined by Drabkin's cyanmethemoglobin method. The equivalent total hemoglobin concentration was calculated for each different dilution.

Table II shows the results of this experiment. The maximum range from the average of the density ratios is $\pm$ 0.003. On our instrument this is equivalent to only $\pm$ 0.6% methemoglobin, while the total hemoglobin concentration varies from 10.0 to 17.5 g. per 100 ml. of whole blood. This provides evidence that the method adequately compensates for the usual variation in the amount of total hemoglobin. Moreover, the demonstrated precision (Tables I and II) validates the use, in this procedure, of an extended absorption scale beyond what is usually considered the effective range.

TABLE II

Dilution	Equivalent g. Total Hemoglobin Per 100 ml. Whole Blood	0% METHEMOGLOBIN			100% METHEMOGLOBIN		
		D_{520}	D_{620}	D_{520}/D_{520}	D_{520}	D_{620}	D_{520}/D_{520}
1—81	17.57	1.155 1.155	0.100 0.097	0.086 0.084	1.155 1.155	0.602 0.602	0.521 0.521
1—101	14.0	1.000 1.000	0.086 0.084	0.086 0.084	1.000 1.000	0.523 0.523	0.523 0.523
1—121	11.72	0.854 0.839	0.074 0.071	0.084 0.082	0.854 0.839	0.444 0.438	0.520 0.522
1—141	9.99	0.745 0.745	0.064 0.066	0.086 0.088	0.745 0.745	0.387 0.387	0.519 0.519
Average	....	....	....	0.085	....	....	0.521
Range	....	....	....	0.082–0.088	....	....	0.519–0.523

COMMENT:

The usual precautions for photometric work must be scrupulously observed. Instrument voltage must remain constant for consistent readings. Each instrument used for this determination must be calibrated by the procedure for standardization. Rarely are the constants found identical for different instruments of even the same make and model

The standardization must be checked when any repairs or replacements are made on an instrument, or when instrument trouble is suspected.

Most clinical photometers are equipped with the 520 mμ filter, but it is usually necessary to add the 620 mμ filter. This is easily done at a reasonable cost. It is important to use the specified filter. On instruments equipped with monochromators or diffraction gratings, their operation must be regularly checked. Substitution of inaccurate wave lengths can lead only to the confusion of erroneous results.

The procedure should be followed exactly to maintain accuracy and replicates must conform within a proper range of precision. If it is absolutely necessary to use an anticoagulant, heparin is the one of choice. The use of fresh whole blood, with no anticoagulant, is preferable. The use of oxalates must be avoided. They contribute to the formation of alkaline methemoglobin, which has different transmission values and different isobestic points with oxyhemoglobin. The photometric readings must be made immediately after centrifugation, as the dilutions will become cloudy again after standing. The entire procedure should be completed within two hours after taking the blood specimens. Alterations of the components may occur spontaneously after prolonged delays.

In the calculations for standardizing, it is wise to retain the decimals until the constants have been determined. Otherwise, accuracy is lost. In the final equation for unknown values, the decimal places in the constants may be disregarded for all practical purposes. Values of the densities and of the density ratios should be correct to three decimal places. Greater arithmetic accuracy has no clinical significance.

CONCLUSION:

A modification of the methemoglobin method of Hamblin and Mangelsdorff is presented. Optical densities of methemoglobin and oxyhemoglobin, in a common solvent, are measured at two different wave lengths. The relative proportion of the methemoglobin pigment is calculated from the absorption ratio. In addition to the technical procedure, the theory of the principle is developed algebraically from the basic concepts of the Bouguer-Beer law.

Our own experience, using three different instruments, has provided evidence of adequate accuracy and precision for clinical work. This modification requires a very small blood sample, is simple and rapid, and utilizes instrumentation commonly available in a clinical laboratory.

ACKNOWLEDGMENT:

Grateful acknowledgement is hereby expressed to Dr. D. O. Hamblin and Dr. A. F. Mangelsdorff for their kind permission to modify their original procedure.

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AMONG THE NEW BOOKS

CHOLESTEROL by David Kritchevsky, Associate Member, The Wistar Institute, Assistant Professor of Biochemistry in Medicine, The University of Pennsylvania. John Wiley & Sons, Inc., New York, N. Y. pp. 291. 1958. $9.75. Tables.

Supplementary information for the biochemist on the biological function and significance of cholesterol. The writer states that he gathered and organized the material in order that the usual store of information might be found in one place rather than scattered throughout the literature, for a "background for future investigation."

THE PRACTICAL USE OF THE MICROSCOPE Including Photomicrography. By George Herbert Needham, M.S., F.R.M.S., Past President of the New York Microscopical Society, Past President of the San Francisco Microscopical Society (Reorganized 1946), Lecturer in Microscopy and Photomicrography, University of California Medical School, University Extension, San Francisco, California, Consultant in Microscopy and Photomicrography. Charles C. Thomas, Publisher, Springfield, Illinois. 1958. 493 pp. 173 illustrations. 48 micrographs. $15.50.

Microscopy brought up to date—the various types of microscopes are described, applications, principles of use, enough theory to enable the reader to interpret the practical directions, and proceeding from the simple to the complex makes this a valuable addition to the teaching library.

Chapters and sections on microscopes from Simple, Compound, The Binocular, Wide Field, Stereoscopic Binocular, Fluorescent, Electron, Ultraviolet, Phase Contrast, and the Polarizing Microscopes illustrate the wide field covered. Principles involved, and types of parts—eyepieces, substage condensers, objectives—all have chapters devoted to descriptions and detailed matter. Still other chapters include information on Micrometry, Mounting Media for specimens, and Microprojection. There is a 52-page section on Photomicrography.

BUILDINGS FOR RESEARCH, an Architectural Record Book, by the editors of Architectural Record, published by F. W. Dodge Corporation, New York, N. Y. 1958. pp. 224. 500 photographs. $9.50.

A selection of outstanding laboratory designs—research laboratories, smaller units within the hospital, industrial concerns—for nuclear, biological, electronic, or chemical research. The first section, on *Planning the Laboratory* has much information that will apply to the design necessary to provide, not only adequate, but outstanding, laboratory facilities. A far cry from the time, less than a decade ago, when the laboratory was located in some otherwise unused space, no matter how inadequate, of a building designed for other purposes primarily. The principles may well be applied to the routine clinical laboratory.

A SIMPLE FREEZE-DRYING APPARATUS*

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Freeze-drying techniques offer advantages for the preservation of certain biological materials, but limited budgets often prohibit the purchase of commercial equipment. We have devised a simple lyophilization apparatus, to be used in our laboratories for the preservation of bacterial cultures, which can be completed by others at very reasonable cost.

Fabrication Procedure

Cylindrical stainless steel beakers, straight wall type, with turned lips, but no pourouts, were used as components for the apparatus. These are available in appropriate sizes (Table 1) from a number of laboratory supply houses.

TABLE 1

Beaker Sizes Used as Apparatus Components

Component	Beaker Capacity in ml	Beaker Diameter in mm	Beaker Height in mm
Main unit condenser	2000	123	170
Main unit jacket	5750	183	218
Trap condenser	1200	105	145
Trap jacket	2900	155	183

The beaker used as the outer jacket for each unit of the apparatus was first drilled at predetermined points from the inside, to form flared openings to which could be attached (on the outside) one and one-half inch lengths of stainless steel tubing. Considerable care was necessary in this operation to avoid cracking the metal around the openings. Block tin solder was used to join a length of tubing to each flare, thus forming a vacuum tight seal. Five-eighths inch diameter inlet and outlet ports were constructed on the unit which was to serve as a trap, as was a single inlet port on the main lyophilization unit. The other twelve ports on the main unit, as shown in Figure 1, were constructed of three-eighths inch diameter steel tubing.

Once the ports had been attached to the larger beakers, which were to serve as outside jackets for the respective vacuum chambers, the inside beakers were attached to comprise the inside walls, or condensing surfaces. Outer and inner walls were joined at the top by a collar of stainless steel sheet, which should be two or three times the thickness of the beaker walls. The collar was welded along its outer edge to the lip of the outside beaker, and along its inner edge to the lip of the inside beaker. A Heliarc welding tip was used, so that only the metal being joined served to form the weld.

The completed units were finally connected by means of rubber vacuum tubing between their lower five-eighths inch ports. The twelve smaller inlet ports, to be used for attaching samples for lyophilization,

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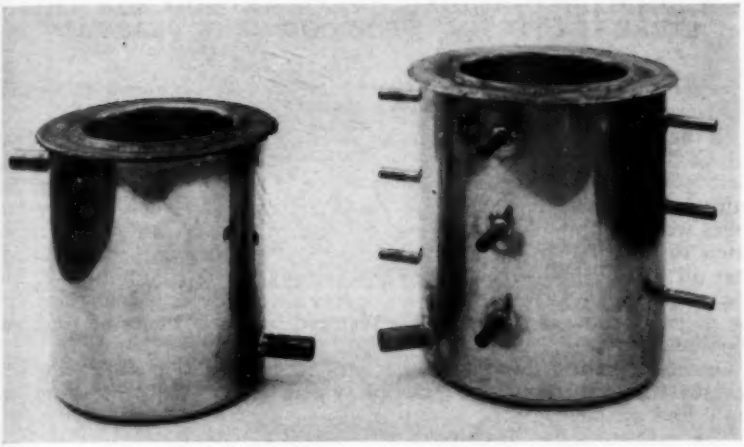

Figure 1. Trap and main lyophilization units welded and soldered.

were fitted with three inch lengths of rubber vacuum tubing, and plugged with short lengths of glass rod.

In actual operation, the condenser beaker of each unit was filled with dry ice and ethanol, and covered with a wood or rubber bung. The trap was attached to an efficient vacuum pump, and measurement of the vacuum was made on a McLeod or Stokes gauge. When the vacuum measured approximately 50 microns of mercury or less, a sample was attached to a port by first clamping the rubber tubing with a hemostat, removing the glass-rod plug, and replacing with the sample tube. The sample, of course, was first frozen by dipping it in the center pool of alcohol and dry ice.

Acknowledgment

The authors wish to acknowledge the contributions of Mr. I. A. Coleman and the staff of the Iowa State University Instrument Shop in the construction of the apparatus.

A SIMPLIFIED AND ACCURATE METHOD FOR THE STERILITY TESTING OF BLOOD BANK PLASMA***

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In the Bacteriology Laboratories of the University of Arkansas Medical Center, plasma pooled by the Blood Bank is tested for sterility. Cotton plugged thioglycolate tubes (3 per sample) had been used for culturing the plasma. There were many occasions when one or two tubes were contaminated. The use of three tubes per sample was instituted to eliminate the factor of air contamination on the assumption that all tubes should have growth if the plasma is contaminated. The pooled plasma was re-checked for safety's sake even if one tube of a set of three showed growth. This resulted in much waste of time and supplies.

On investigating this problem it was found that a method could be devised which incorporated a closed system and at the same time was rapid and simple.

The present paper describes:

1. The method of preparation of a completely closed system for testing the sterility of plasma.
2. The equipment necessary for this procedure.
3. The results up to the present time at the University of Arkansas Medical Center.

Materials and Methods

The necessary equipment may be found in most hospital pharmacies which prepare any of their own solutions.

Bottle—We find it convenient to use a 250 ml U.S.P. Type 1 glass "No-Sol-Vit" glass bottle.* Cost, approximately $1.00 per bottle.

Stoppers—Solid rubber stoppers #C-202** Cost, approximately 3 cents each.

Seal—A 43 mm. 3-piece aluminum seal is used.† Cost, approximately 5 cents per seal.

Crimping Machine—T. C. Wheaton "Fernpress" Machines of this type cost about $300.00 but are usually part of the equipment of the hospital pharmacy.

Preparation of Flasks:

125 ml of fluid thioglycollate (BBL) without indicator (to eliminate toxicity of the indicator) is placed into the flasks. This amount gives sufficient ratio of surface to volume (15 ml per ¾" surface.) The bottle is stoppered with a clean rubber stopper and sealed with a 3-piece aluminum seal, using a hand operated crimping machine. The thioglycollate bottles are then sterilized in the autoclave for 30 minutes at 120° C and incubated at 37° C overnight. After the sterilization is completed, the door of the autoclave is opened but the bottles are not taken out for at least ten minutes. (They are allowed to cool to avoid the possibility of explosion.)

†Alcoa.
*T. C. Wheaton Co., Millville, N. J.
**West Co., Phoenixville, Pa.
***Received for publication October 1959.

Figure I

Technologists introducing plasma into thioglycollate bottles. This may be done by one person by placing the plasma bottle in a ring stand with holder.

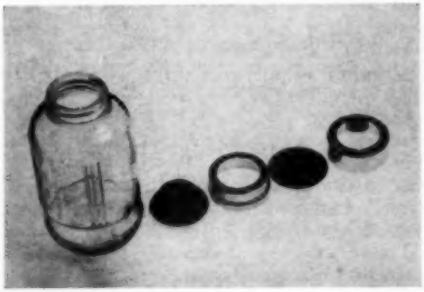

Figure II

Bottle stopper and 3-way seal used for closed system sterility testing.

Inoculation with Pooled Plasma for Sterility Test:

The bottle of pooled plasma is either held or placed on a raised platform to insure a gravity flow into the bottle with positive pressure. The outer seal of the thioglycollate bottle is torn off, exposing the inside metal cap. A two way aspirating set* is used. One end is placed in the pooled plasma bottle and the needle on the other end of the tube is plunged through the rubber stopper (after the metal cap is taken off placed in a sterile petri dish) which has been sterilized with 70% alcohol and then wiped with sterile cotton. 15-20 ml of plasma from a pool of 500 ml is placed in each of the 3 bottles. The metal cap is placed on the flask again and held in place with masking tape. A set of six may be used to eliminate either unforeseen breakage or cracking of a bottle. They are then incubated at 32° C seven days. Gram stains are made after shaking the bottle whether or not growth is seen.

*(R-32)—Baxter Laboratories.

Results

In testing 50 lots of plasma using the test tube method, at least one tube of a set of three was contaminated at three different times. When the original lot of plasma was checked, it was found to be sterile.

At the present time, using the new method, 60 lots of plasma have been checked for sterility. There was one positive. The plasma was re-checked and found to be contaminated.

Discussion

The closed system method has eliminated the time used in flaming individual tubes and setting up a sterile field. It also avoids accidental contamination during the process. It is simple to perform and is relatively fool-proof. Our staff in the Blood Bank has been very enthusiastic in accepting this procedure.

Summary

1. A method is described for a closed system for use in testing the sterility of blood bank plasma.
2. This method is simple to perform and may be utilized without added expense in most hospitals which have modern pharmacies.

Acknowledgment

The author wishes to thank Doctor William Heller, Chief Pharmacist of the University of Arkansas Medical Center for his many helpful suggestions and access to his equipment.

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THE IMPORTANCE OF ENZYME-CHEMISTRY IN THE CLINICAL LABORATORY*

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Since the earliest times of human history, the action of unknown substances in the making of bread or making of wine and beer was observed, which brought subsistence and happiness, or in the case of alcohol, weariness and stupor to man. These unknown forces, which today we call ferments or enzymes, are becoming more and more of importance in the clinical laboratory.

Their behavior in the human body, that is their presence in the blood stream, becomes an aid in the diagnosis and prognosis of diseases and in following up the effectiveness of the applied forms of therapy.

It is not the purpose of this paper to give an enumeration of all the known enzymes and their behavior in the human body, only some of the newer ones, which seem to us of such importance that they should be included in the clinical routine tests.

Up to 1956 the laboratories of smaller hospitals were concerned only with the determination of a few enzymes for diagnostic purposes. They were:

1) Determination of the serum or plasma *Amylase*. This enzyme is primarily involved in the digestion of starch in the small intestine. High values are found in acute pancreatitis, in salivary gland inflamation, in mumps, in carcinoma of the head of the pancreas, in duodenal ulcers, and in intestinal obstruction. Low values are found in liver diseases and pancreatic insufficiency.

There are different test methods,[7,36] but they are based mostly upon the hydrolysis of starch by the enzyme under standard conditions of pH, temperature and time, and the calculation of the reducing sugar (Maltose) which is formed.

In some cases the determination of urinary amylase may be helpful for prognostic purposes, since the enzyme activity in cases of acute pancreatitis will be found elevated for a longer time in the urine than in the blood.[37]

2) The determination of *Lipase*. This enzyme is mainly formed by the pancreas and it is an important factor in the hydrolysis of the ingested fats in the small intestines. In pancreatic disorders the enzyme may show elevated values in the blood.
3) The determination of *Trypsin*. This digestive enzyme of the pancreatic juice which hydrolizes the proteins in the stomach into their constituent amino-acids. Since only the smallest amounts find their way into the blood stream, the determination is made on feces or samples of aspirated digestive juice. Decreased levels are mainly found in fibrocystic diseases of the pancreas.
4) The determination of *Pepsin*. This enzyme hydrolyses proteins to proteoses, peptones and peptides in the gastric juice. The hydrolysis to the constituent amino-acids is completed by trypsin in the duodenum. It is never absent in the gastric juice if free HCl is present.

*Read before the 27th Annual Convention of ASMT, Phoenix, Arizona, June 1959. Received for publication February 1959.

5) The determination of *Alkaline and Acid Phosphatases.* These two enzymes received their names according to their optimum pH activity.

The two groups of diseases resulting in an increase of alkaline-phosphatase activity in the human serum (which is most active depending on the methods used at a pH range between 8 to 10) are the diseases of the bones by an increased osteoblastic activity and in liver disorders. The alkaline phosphatase is of a much higher concentration in obstructive jaundice than in non-obstructive cases. Since the osteoblastic activity is 2 to 3 times increased during the period of growth, the clinical evaluation of this enzyme during infancy is difficult.

The acid phosphatase has its major source in the prostatic gland. The phosphate splitting enzyme is active in the acid range at a pH of 5 and, therefore, differs from that of the bone or plasma, where it is only found in small quantities. Besides the determination of the total acid phosphatase a differentiation can be made by a formaldehyde or L-tartrate method between prostatic and non-prostatic acid phosphatase (red-cell acid phosphatase).*

Besides the prostatic phosphatase there are other acid phosphatases found in the tissues of liver, spleen and kidney. While in females the acid-phosphatase usually is present only in very small amounts—below the normal for males—the enzymatic activity is increased in cases of carcinoma of the breast. The source[12, 20, 13] of the prostate-like acid-phosphatase in females is unknown. (A moderately increased acid-phosphatase may be found also in cases of Paget's disease, in acute myelocytic leukemia and hyperparathyroidism.)[12, 20, 13]

This was only a very short review of the enzymes generally used in hospitals up to 1956.

Then a great number of enzymes, known before only in research centers, became of actual interest for the clinical laboratory, and new methods were developed so that the tests may be done in any progressive institution. Time does not allow to evaluate a great number of these tests. Therefore, only a few can be mentioned which should be considered as routine procedures. They are:

1) **The Transaminases,** the Glutamic Oxalacetic Transaminase (GOT), and the Glutamic Pyruvic Transaminase (GPT). A report about these two enzymes has been published.[30] We can, therefore, be very short and summarize only. The GOT is found mainly in the heart muscle, the liver, the skeletal muscle and the kidney, the GPT mainly in the liver and the kidney. This may be helpful in diagnosing some cases. In hepatitis the GOT and GPT will be very much elevated, while in myocardial infarction only the GOT will show a real elevated activity. It will therefore be possible in dubious cases to differentiate between a myocardial infarct and a hepatogenous enzyme increase. In cases of myocardial infarcts the enzyme activity will rise above the normal between the 4th and 6th hour after the attack. It will reach its peak between 24 and 36 hours, and—if no other complication occurs—it will come to a normal (8 to 40 units) after 3 to 5 days.

*(Methods by: Fishman and Lerner, J. Biol. Chem. 200:89 (1953). Bensley, Am. J. Clin. Path. 26, 247 (1956).

May we stress again the importance of the transaminase determination for the blood centers. We found by actual experience—and we have used the test in our blood bank since late 1957—the GOT determination the most reliable test to avoid the transmission of homologous serum hepatitis. The GOT or GPT will show abnormal values 14 to 20 days before any other liver function tests show abnormalities. We will give a summarized report at a later date. It may also be mentioned that in cases of chronic alcoholism an increase will be observed for 1 to 11 days after an acute alcohol intoxication, while healthy persons, after ingestion of large amounts of alcohol, showed no increase.[3]

2) **The Lactic Dehydrogenase** (LD) which is present in the entire tissue system, mainly in the liver, kidneys, myocardium, skeletal muscles and in erythrocytes. The enzyme is found as well in serum or plasma as in serous effusions and cerebrospinal fluid. The first investigation regarding the enzyme activity in pathological human sera were reported by Hill and Levi in 1954.[16] They found an increased activity in serum of cancer patients.

The LD regulates the transmutation from pyruvic acid + Disphosphorpyridinnucleotid + H (DPN red.) to lactic acid + DPN ox., by activation of the H-ions. The normal range in serum ranges from 200 to 680 units per ml., in cerebrospinal fluid 40 units or less. The activity varies depending on the pathological processes in tissues.

As in the case of Serum Glutamic Oxalacetic Transaminase (SGOT) there are large amounts of LD present in the cardiac muscle which are released into the extracellular fluid by any necrosis of myocardial tissue, accounting in a rise of LD-activity by a myocardial infarction.

Wroblewski and co-workers[39, 40] observed that in 6 to 12 hours following the onset of the myocardial infarct a rise of 2 to 10 times of the normal occurred, with a peak in elevation after 24 to 48 hours. The behavior is similar to the SGOT and the range—if no new complications occur—is reached in 7 to 11 days (see figure 1). A rise in the enzyme activity to 3,000 or more units must be regarded as very critical.

While the two transaminases are of greatest value in the diagnosis, prognosis and therapy of myocardial infarcts and liver diseases, the LD-determination amplifies the diagnostic spectrum, since elevated values will be found in cases of carcinoma, leukemias and in anemias. In leukemias and anemias an activity of 10 times of the normal may be found, because the erythrocytes have an enzyme activity which is up to 100 times higher than the one in the serum, the plasma or the leukocytes. In some cases of chronic myelogenous leukemias a relationship between Serum Lactic Dehydrogenase (SLD) and leukocyte count can be established but it is not a consistent observation.[39]

An increased SLD activity is usually observed in disseminated carcinomas and lymphomas. There seems to be a semi-quantitative relationship between the enzyme activity and the tumor growth and spread of the neoplastic process. It is assumed that neoplastic cells liberate LD into the fluid which bathes the cells, which finds its way into the extracellular fluid.

A further increase in LD activity will be found in acute hemolytic episodes, whereby the LD from the erythrocytes is liberated and finds

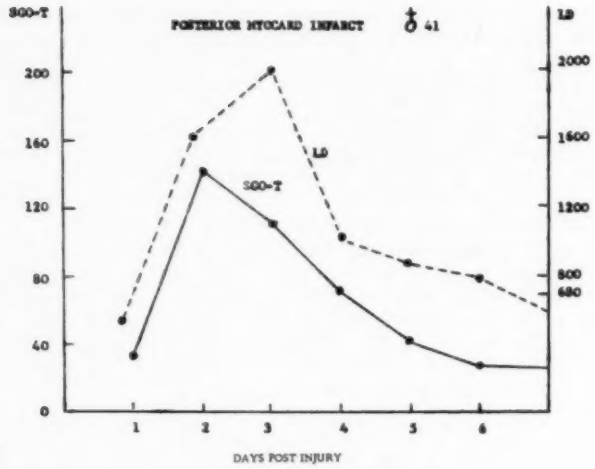

Figure 1

its way into the serum or plasma.

As in the case of SGOT there will be an elevation in SLD observed after surgery for 24 to 72 hours, due to liberation of the intracellular LD into the extracellular fluid.

Since the enzyme activity will be increased in different pathological events it cannot be regarded as a specific test, but it is of greatest interest after a diagnostic evaluation has been made in following the acute stage.

The case is different in testing the enzyme activity in serious effusions (Ef-LD) and in cerebrospinal fluid (CSF-LD), where it is of greater value than the determination of the transaminase activity.[39] "Pleural and peritoneal effusions containing malignant cells may be conceived of as in Vivo cultures of cancer cells."[39] In an effusion, associated with a non-neoplastic disease the enzyme activity will be found lower than in the serum or plasma of the same individual. But the enzyme activity in effusions containing malignant cells is usually much greater than the enzyme activity in serum or plasma of the same individual. This may be the result of the enzyme being primarily a globulin component and the globulin fraction of an effusion does not exchange readily with the plasma constituent (see figure 2).

It was also observed that in serious effusions of patients with a malignant neoplasia, in which no malignant cells were found, that the enzyme activity was lower than in the serum. Wroblewski[39] concludes, therefore, that the findings of the cytologic examination and the enzymatic activity of a serous fluid are two related but, nevertheless, potentially independent expressions of malignant neoplasia within a serous cavity. These findings need further study before a definitive conclusion

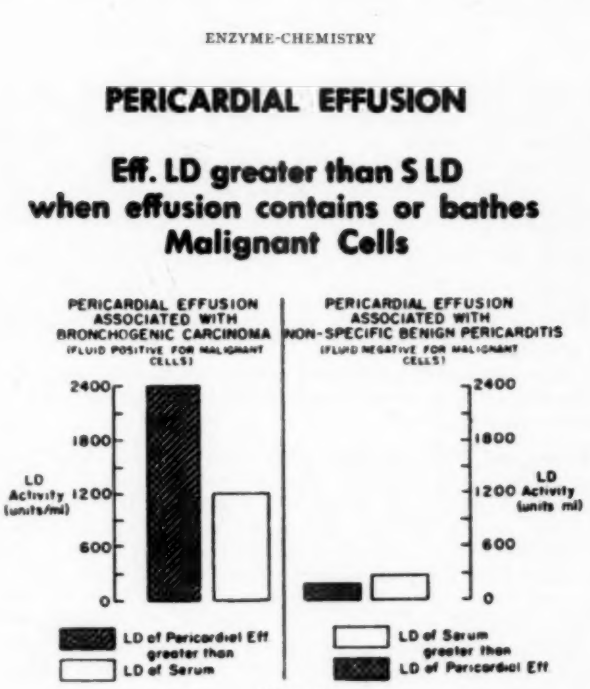

Figure 2
This illustration compliments of Dr. F. Wrobleski.

can be reached as to whether this test can be regarded as specific for the characterization of effusions.

It was established that the LD-activity in the spinal fluid of healthy persons and free of any central nervous system disease will always show lower values than those in serum or plasma. The normal range was established as below 40 units per ml. In contrast, the activity was above 40 units in cases of intracerebral or meningeal leukemia, lymphoma and metastatic carcinoma with central nervous system involvement, than in cases of untreated meningitis, and in cases of subarachnoid hemorrhages. The mechanism by which the enzyme activity in the spinal fluid becomes increased is not entirely clear. It is assumed that in pathological states in which blood or plasma reaches the CSF the enzyme from the plasma brings the increase in the CSF. In cases of metastatic carcinoma, lymphoma or leukemia, the increased enzyme activity in the CSF may be the result of malignant tissue cells, as it was found in cytologically malignant effusion.[39]

The original procedure for the determination of the LD-activity requires spectrophotometers measuring in the range of 340 mu., whereby the change in optical density of DPNH was determined.[40]

Two new colorimetric methods, which are accurate and can be performed with standard equipment, are available. The one developed by Gabaud and Wroblewski[11] measures the LD activity of serum, serous

effusions and cerebrospinal fluid by the reduction, in the presence of DPNH, of pyruvic acid to lactic acid by a wave length of 550 mu.

The method of Berger and Broida[8] is based on the same theory and can be determined at a wave length between 500 to 550 mu. This method has the advantage because the unstable DPNH solution is available in a dry stabilized form. For smaller laboratories this method is to be recommended.

Undiluted sera can be stored in the refrigerator for three days, or frozen for ten days, without significant loss of LD activity.

Instead of serum, heparinized plasma can also be used. Plasma from oxalated blood cannot be used, since the oxalate apparently inhibits the LD actvity.

The slightest hemolysis will give erroneous results, since the red cells contain an LD activity which is up to 100 times higher than the activity of serum or plasma.

3) **Cholinesterase.** This enzyme splits acetylcholine, the chemical transmitter of the nerve impulses. It was first discovered by the Austrian physiologist Loewi.[18] There are two types of this enzyme known. The one, found in the nervous tissue and the red cells, hydrolyzes only acetylcholine and is, therefore called the "true" cholinesterase. The other, found in the plasma and other body tissues, hydrolyzes not only acetylcholine, but also a number of other esters derived from the aliphatic alcohols, and it is called "pseudo" cholinesterase.[21]

The enzyme activity is reduced—or inactivated—by the alkaloids, eserine, prostigmine, atropine, also by certain nerve gasses, and by organophosphorus insecticides.

Since the enzyme is produced in the liver, the enzyme activity may serve as indicator in liver diseases. In albuminuria the high enzyme activity indicates an increased formation of albumin, and low values a depressed rate of formation.

The red-cell-CHE may be decreased in cases of pernicious anemia and it is said to be indicative of the red cell formation.[26, 27, 31]

A very simple and convenient electrometric method was developed by Michel.[23] The principle is the measurement of acid produced as the result of the hydrolysis of a substrate (acetylcholine) in a measured time, with a pH meter, able to permit readings in units as low as 0.01. The results are expressed as "pH-units" equal to the drop in pH $\times$ 100. The normal red-cell-CHE is in the range of 60 to 100 units, for the plasma-CHE 60 to 120 pH units per hour. In anticholinesterase poisoning (certain insecticides) both values for plasma and red cells can go down to 5 pH units. A rapid screening test for the cholinesterase activity was developed by Limperos and Ranta.[17]*

4) **Phophohexose Isomerase.** This enzyme, belonging to the glycolytic group, catalyzes the reversible reaction of glucose—6-phosphate $\longleftrightarrow$ fructose—6-phosphate. It is found mainly in the skeletal muscle and the liver where it is involved in the metabolism of glucose from glycogen and is present in the serum. Hemolized serum

* A practical test kit is supplied by Biological Test Products Corp., Roselle, New Jersey.

cannot be used, since the enzyme activity is about 160 times higher in the erythrocytes. The enzyme was first discovered by Lohmann[19] in 1933 and a practical method was developed by Bodansky, who proved, for the first time, the enzyme in human blood, in 1954.[6] He also established values in healthy persons with a range between 15 to 40 units. The units represent the reciprocal of the serum concentration expressed as ml. per ml. of reaction mixture necessary to form 25 mcg. of fructose-6-phosphate from 0.002 M glucose-6-phosphate in 30 minutes by a pH of 7.4 and a temperature of 37° C. Bodansky confirmed, also, the earlier findings that the enzyme activity is increased in some metastatic tumors, and that there is a correlation between the enzyme activity and the tumor growth.

In cases of metastatic cancer of the breast, there is an impressive correlation between the growth of secondary tumors in the bone and the increased enzyme activity in the serum. In these cases an increase in urinary calcium will be observed. In cases of tumor of the liver there will be an increased enzyme activity, but without increase in urinary calcium. Cases of carcinoma of the prostate with metastasis to the bone will show also an enzyme increase. The determination of this enzyme, in connection with the determination of the two phosphatases, may be a helpful index of the growth or regression of metastasis in the bone, secondary to the carcinoma of the prostate.

Since the enzyme is found also in the liver, there is always an increased activity in acute hepatitis. Normal values will be found in cases of obstructive jaundice. For this reason the test has its value, too, for differential-diagnostic purposes.[9,10] An increase will be observed also in myocardial infarcts.

Bodansky's[6] method can be done by using any standard colorimeter at a wave length of 490 mu.

5) **Aldolase:** After the formation of fructose-6-phosphate by the action of the enzyme phosphohexose isomerase, an extra molecule phosphate is added to yield fructose-1, 6-phosphate. By the action of the enzyme aldolase this fructose diphosphate is broken up into a molecule glyceraldehyde phosphate and one molecule dihydroxyacetone phosphate. This enzyme was first discovered by Meyerhoff and Lohmann in 1934.[22] The first proof of the existence of aldolase in the serum of rats was done by Warburg and Christian in 1943.[38] They found the main source of the enzyme, if it is increased in the serum, in the skeletal muscle. In the human serum it could not be established for a long time, only in cases of advanced carcinoma of the prostate.[2]

Besides the skeletal muscle, the enzyme is found in greater quantities in the liver, the erythrocytes and the heart muscle.

Under normal conditions, the enzyme activity in human serum is quite constant. The enzyme activity is found to be elevated in conditions associated with an acute cellular necrosis.[32] Any acute tissue destruction results in the liberation of the intracellular enzyme into the blood plasma, and there is a certain proportion between the tissue necrosis and the increase in aldolase activity in the serum.

Depending on the methods used,[15,34,8] the normal range is between 2

to 10 units, each unit being considered as equal to 1 microliter per milliliter of serum per hour.

Of greatest value is the aldolase determination in the difficult diagnosis of a progressive muscular dystrophy,[28,1] also in other diseases involving degenerative changes in muscle and brain tissue.[33]

The enzyme determination will be valuable in cases of prostatic carcinoma, where values between 12 to 24 units are found. Estrogen therapy —if effective—will cause a decrease into the normal range. The test proves in some cases as more reliable than the acid-phosphatase.[2]

6) **Copper Oxidase:** The diagnosis of hepatolenticular degeneration (Wilson's disease) rests upon the demonstration of an increased urinary excretion of copper, amino acids, and a low serum seruloplasmin (copper oxidase) level. There are different determinations,[28,2] but they are time consuming and difficult to perform. Quite often the disease is so far progressed that it is too late for an optimal therapy. A considerable decrease in copper oxidase activity is observed consistently in hepatolenticular degeneration.[4] It is occasionally observed in nephrosis.

A rapid colorimetric test for the enzymatic activity of copper oxidase was developed by Ravin in 1956.[24]

Normal serum shows an extinctive coefficient between 0.150 to 0.200 by a dark lavender color. Abnormal sera produce a pale flesh color.

The sequence of the development of the characteristic biochemical abnormalities in hepatolenticular degeneration is unknown.

Ravin's method is a good screening test for detecting an early stage of hepatolenticular degeneration.

7) **Leucine Aminopeptidase:** This enzyme which is found in all human tissues has its main activity in the duodenum, the kidney and the liver. The intracellular function is unknown, but it is assumed that it is involved in the transfer of L-leucine from one peptide molecule to another and that it is active in the terminal phases of protein digestion.

This enzyme may become an important factor in the diagnosis of cancer of the pancreas, after an intensive study of Rutenburg and coworkers[25] established the fact that urinary and serum leucine aminopeptidase activity was significantly increased in cases of cancer of the pancreas. Increased serum and urinary levels appear to be of value in the early diagnosis of the disease, since they have been found in patients without symptoms referable to the cancer, without jaundice, and with normal serum alkaline phosphatase. An increase of enzyme activity in the urine was also established in cases of malignant lymphoma, leukemia and related disorders by normal or only slightly elevated serum levels.

A practical colorimetric method for the determination of the enzyme activity in serum and in urine was developed by Goldberg and Rutenburg.[34]

Summary

It is known today that all vital processes depend more or less on the activity of the enzymes. They are of greatest importance in the diagnosis, in the prognosis and in the establishment of the therapy progress.

This paper is a short compilation of only some of the large number of bio-active matter which has entered in the last years into the realm of clinical procedures out of the field of purely research.

A summary illustrating the importance of enzyme chemistry is represented in the following chart:

ENZYMES IN MODERN MEDICINE FOR DIAGNOSIS AND PROGNOSIS

Prostatic, Breast Cancer, Cancer of Pancreas	Malignant Lymphoma or Leukemia	General Bone Diseases	Hepatic Diseases	Myocardial Infarction
Acid Phosphatase, Aldolase, Isomerase, Leucine Aminopeptidase	LD Leuc. Amino-peptidase	Alkaline Phosphatase	SGPT, SGOT, LD Alkal. Phosphatase Copper Oxidase	SGOT LD
Digestive Enzymes in small intestines, stomach and pancreas.		Central Nervous System	Muscul. Breakdown	Insectcide Poisoning
Trysin, Pepsin, Amylase, Lipase		LD	Aldolase	Cholinesterase

Acknowledgment

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ACID HEMATOXYLIN STAINING OF CONNECTIVE TISSUE ELEMENTS: The Effects of Unripened Linder-Thomas Phosphomolybdic Acid Hematoxylin on Reticulin and Basement Membranes Using Routine Methods of Fixation*

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Introduction

A phosphomolybdic acid hematoxylin lake was described by Thomas in 1943 for the staining of reticulin and collagen. Linder, in 1949, discovered by chance that upon ripening, the basement membrane of glomerular capillaries can be observed.[1] The method, as described, calls for ten weeks ripening of the acid-dye lake. The purpose of this paper is to determine the usefulness of this technic for routine fixation, and if ten weeks ripening is required.

Materials and Methods

Slices of human lung were fixed in Bouin's solution and neutral formalin (10%). The pieces were embedded in "tissuemat" (paraffin with a latex base), sectioned at five microns and mounted on glass slides. One section of Bouin fixed and two sections of formalin fixed tissue were hydrated for each staining period. The staining period ran at approximately ten day intervals, using one day old to fully ripened phosphomolybdic acid hematoxylin.

The staining solution was prepared by dissolving 2.5 Gm. hematoxylin in 49 ml. dioxan and 1 ml. distilled water, as one solution. A second solution containing 16.5 Gm. phosphomolybdic acid in 44 ml. distilled water and 11 ml. of ethylene glycol was made and filtered. Equal volumes of the two solutions were mixed and allowed to ripen at room temperature.

A counterstain with 0.02% Acridin Orange in 1 per cent acetic acid was used.

Procedure:[2]

1. Sections are hydrated to distilled water by the usual graded ethanol technique.
2. Place slide in Lugol's iodine solution ($I:KI:H_2O = 1:2:100$) for one (1) minute.
3. Five (5) percent sodium thiosulfate for one (1) minute.
4. Wash slides in distilled water.
5. Barely cover sections with phosphomolybdic acid hematoxylin and stain for ten (10) minutes.
6. Wash with distilled water.
7. Counterstain with acridin orange for five (5) minutes.
8. Dehydrate, clear and mount with a resinous medium.

Results: Collagen, reticulin and basement membranes of pulmonary capillaries, deep violet; red corpuscles, purple; nuclei, blue, but lightly stained; cytoplasm, bluish. Elastic fibers in unripened sections stain orange to yellow. Results of slides compared to time of ripening on Bouin's fixed tissue are given in table I; on neutral 10 percent formalin,

* Scientific Products Foundation Award, 3rd Histology, 1959. Received for publication February 1959.

in table II. Formalin fixed tissue yields better results, generally, than Bouin's fixed tissue.

TABLE I: Bouin Fixation

SUBSTANCE STAINED	TIME						
	20 Hours	10 Days	20	30	42	55	Fully Ripened
Collagen	++	++	+	+	+	++	+
Elastic fibers	++	—	—	—	+	—	—
Reticulin	+	+++	+	++	+++	++	+++
Epithelial Basement Membrane	++	+++	+++	++	+++	++	+
Blood vessel basement membrane	++	++	+	++	++	++	+
Counterstain	+++	++	+	+	+	+	+

TABLE II: Formalin Fixation

SUBSTANCE STAINED	TIME						
	20 Hours	10 Days	20	30	42	55	Fully Ripened
Collagen	+++	++	++	++	++	++	+
Elastic fibers	++	—	—	—	+	—	—
Reticulin	+++	+++	++	++	+++	++	+
Epithelial basement membrane	+++	+++	++	+++	+++	+++	+
Blood vessel basement membrane	+++	++	++	+++	+++	+++	+
Counterstain	+++	++	+	+	+	+	+

KEY: — = indistinguishable.
+ = distinguishable, but poorly stained.
++ = fairly stained.
+++ = stained well.

Discussion and Comment

Upon ripening, the staining solution became more viscous and more difficult to wash from the slide. The incomplete washing left stain fragments on the tissue section, often obscuring detail and vision. To alleviate this problem the tissues were washed under tap water, and rinsed with distilled water before proceeding.

The optimal ripening time seems to be between one day and two weeks, after which, details within the elements become diminished. The elastic fibers were apparent only while the stain was comparatively fresh. The counterstain lost significance after two weeks ripening because it was obscured by the hematoxylin stain. The method could be applied to routinely fixed tissue if the stain were not more than two weeks old. There is value in the staining technic in that it shows all connective tissue elements in a one-step operation.

Summary

An evaluation of the Linder-Thomas Phosphomolybdic Acid Stain was made varying the time of ripening on two routine fixatives. The results showed that the best staining is with formalin fixed tissue when the stain has not ripened more than two weeks.

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CURRENT EDUCATIONAL STANDARDS IN MEDICAL TECHNOLOGY*
(Consideration of the Bachelor of Science in Medical Technology Degree)

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Historical writings and recruitment literature[36, 37, 38] tell us that Medical Technology is a relatively new science, which was born following World War I. It came of age after World War II, and now stands upon the threshold of an unlimited future in the vast field of Medical Science.

The development and progress of the science of Medical Technology has been a difficult uphill battle. Innumerable technical, scholastic, and public opinion problems were encountered, studied, solved and largely overcome.[3, 4, 5, 6]

The educational standards for medical technologists have improved greatly since the profession has become recognized and accredited as a primary Science.[8-16, 21-34]

1. Before WWI there were no clinical pathologists or medical technologists. Doctors did their own laboratory procedures, etc.
2. During WWI doctors and laboratory workers were trained for laboratory procedures and blood transfusion techniques.
3. The American Society of Clinical Pathologists was organized in 1924.
4. The A.S.C.P. in 1926 appointed a Committee to study and develop standards for medical technologists.
5. In 1928 the A.S.C.P. reorganized the above study committee and named it the Board of Registry of Medical Technicians of the A.S.C.P. The name was subsequently changed to Registry of Medical Technologists of the A.S.C.P. in 1937.
6. From 1929 to 1933 this Board certified practicing laboratory workers, duly recommended by pathologists as registered laboratory technicians. No examinations were required.
7. Beginning in 1933 all applicants for the Registry were required to pass an examination before certification. Applicants were required to have a minimum of 30 semester hours of college credits including 8 semester hours of college chemistry and 8 semester hours of college biology.
8. On August 29, 1936,[18] a "Survey of Schools for Clinical Laboratory Technicians" was published in the J.A.M.A. This survey was conducted by members of the Council on Medical Education and Hospitals. It was informally requested by the Board of Registry of Technicians of the American Society of Clinical Pathologists in 1933. The members of the Council staff visited one hundred and ninety-six schools. They were of three general types:

   (1) The College or University. These offered generally standard collegiate courses the fourth year of which was given over to instruction and practice training in a hospital laboratory department affiliated with the education institution. As a rule, on the completion of the fourth year, a Bachelor of Science Degree in Medical Technology

*Based on a paper presented at annual meeting of Mississippi State Society of Medical Technologists, April, 1958. Received for publication in the AJMT, November, 1959.

was conferred. There were twenty-six such schools.

(2) Hospital Laboratory Department. Courses were conducted on the Apprenticeship Basis. These hospital schools admitted students with from one to four years of prerequisite collegiate preparation, or graduates of an accredited nursing school. This group included one hundred and forty-nine hospital laboratory schools, and twelve independent laboratories.

(3) Commercial Schools. There were nine schools in this category. Because of the necessarily large tuition, extravagant advertising, follow-up letters, promises of placement, and acceptance of students regardless of preparation, this type of school was not considered desirable and consequently was not included in types of acceptable schools.

This same publication included a list of the "Schools for Clinical Laboratory Technicians Conforming to the Standard Adopted by the American Medical Association in 1936." Also recorded were the "Essentials of an Acceptable School for Clinical Laboratory Technicians." The essentials were adopted by the Council on May 10, 1936, and after having been approved by the Board of Registry of the American Society of Clinical Pathologists were passed by the House of Delegates at the Kansas City session May 11-15, 1936.

The approval or recognition of the first two types of training programs for Clinical Laboratory Technicians continued until June 4, 1941,[39] when the Council on Medical Education and Hospitals of the American Medical Association with the cooperation of the American Society of Clinical Pathologists released a revised version of the "Essentials of an Acceptable School for Clinical Laboratory Technicians." This announcement stated that acceptable schools for training laboratory technicians may be conducted by general hospitals, or state health laboratories affiliated with hospitals, where the majority of the students practical training is received. It continued that this arrangement should not discourage affiliations between the hospitals and universities, colleges, public health laboratories or other hospitals. By this ruling, the universities and colleges formerly listed as approved schools for Clinical Laboratory Technicians became the affiliates of the various hospital training schools associated with them. At the time of this change there were about thirty-three colleges and universities affected.

9. In 1933 the requirements for entrance into an approved Hospital Technical Training School of Medical Technology were:
   a) Graduation from an accredited high school, or an equivalent education, a minimum of one year of college work, including Chemistry and Biology.
   b) Graduation from a School of Nursing recognized by the State Board of Nurse Examiners.

   The approved hospital technical training for both (a & b) was for a period of twelve consecutive months.
10. On January 1, 1938 the collegiate requirements were raised to two years. This program continued the required twelve month hospital technical training.[19, 20, 43]

11. In 1930, Dr. Hillkowitz,[2] then Chairman of the Board of Registry of Technicians of the A.S.C.P., defined the usage of the terms medical technologist and laboratory technician. He stated that the term medical technologist would designate one who possessed a university degree with at least one year of practical experience in a recognized laboratory. Laboratory technician is an old term used to designate the experienced laboratory worker who was not college trained. This term was officially discontinued in 1936.

At the present time there are various grades of recognition for laboratory workers, but the title of Medical Technologist is still reserved for those scientists who have had prerequisite collegiate course work prior to their hospital technical training.

For several years a real need for a minimum of three years preclinical training, or pre-hospital technical training has been recognized by the leaders in the field of Medical Technology education. Now, through the combined efforts of these interested groups a three year collegiate program will be prerequisite to the year of hospital technical training effective January 1, 1962. [17, 41, 42, 44]

In 1957, Miss Thelma Wilson, M.T. (ASCP), then Chairman of the Standards and Studies Committee of the American Society of Medical Technologists sent a questionnaire to 550 directors and/or administrators of Approved Schools of Medical Technology. The questionnaire requested information concerning preferred subjects to be taught at the collegiate junior year level prior to the year of hospital technical training.

While answers did not include sufficient information to list the number of semester hours for recommended courses, nevertheless, sufficient material was submitted to record in percentage figures, the collegiate courses most desired by the Pathologist Directors of approved Hospital Technical Training Schools of Medical Technology. The results are listed below:

1. General or Basic Bacteriology	61%
2. Physics	53%
3. Quantitative Analysis	46%
4. Biological or Physiological Chemistry	43%
5. Medical Bacteriology	37%
6. Parasitology	35%
7. Organic Chemistry	35%
8. Mathematics	35%
9. Physiology and/or Anatomy	29%
10. Histology	20%
11. Physical Chemistry or just Chemistry	13%
12. Immunology	12%
13. Mycology	10%
14. Hematology	9%
15. Zoology	7%
16. English	5%
17. Genetics	5%
18. Miscellaneous subjects: (languages, embryology, bloodbanking, ethics, etc.)	28%

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10. Histology	20%
11. Physical Chemistry or just Chemistry	13%
12. Immunology	12%
13. Mycology	10%
14. Hematology	9%
15. Zoology	7%
16. English	5%
17. Genetics	5%
18. Miscellaneous subjects: (languages, embryology, bloodbanking, ethics, etc.)	28%

The commentaries of the School Directors on the above listing were interesting and valuable (sometimes surprising). The references to Bacteriology, general and medical, indicated a strong desire for them, however, doubt was expressed as to the feasibility of their inclusion in the collegiate curriculum. Many of the Directors stated that Medical Bacteriology would be preferable to General Bacteriology; some wished that it could be taken immediately after the General Bacteriology course. It is interesting to note that many of the Directors stated that unless the courses were given by an M.D. or an M.S. in Bacteriology, they would be of little value.

Some Directors indicated that Parasitology should, or could, be taught as a part of the Bacteriology course.

Grouping the comments on the Physical Sciences, it was found that many of the Directors requested serious consideration of good foundation studies in mathematics (some even advocated courses as advanced as Calculus). With reference to Chemistry, it appeared that there is a growing appreciation of this important aspect of the pretechnical training of the student Medical Technologist. Comments on the science of Physics showed little interest in the typical general course, but indicated a genuine approval of courses in instrumentation.

Miss Wilson stated that the committee's definite conclusion, after going over the replies, "was that at least ¾ of those replying felt a Medical Technology student should have a better college background in theory of chemistry, bacteriology, instruments, physiology and mathematics, so that his period in the hospital would be more of an actual training period, applying his theory to technical practice. It was felt that under present conditions too much time had to be spent on teaching the student to do the many tests needed, satisfactorily, and to correlate these tests to his college training."

This survey brought fourth the idea that the entire three year course should be reevaluated and revised rather than just adding requirements to make a third collegiate year. One hard-to-believe fact revealed by the survey was that fourteen Directors opposed a three year pretechnical program because it would cut down on the number of applicants for hospital training schools for Medical Technologists. This attitude shows shortsightedness of the seriousness of the problem of training Medical Technologists to keep abreast of the ever expanding and developing field of Medical Sciences. The Medical Technologist should not be stymied or sidetracked from pursuing the best program which will offer the proper foundation for his profession. The Medical Doctor after his internship must spend several years in residency before he is allowed to assume the responsibility of his chosen specialty field. In the field of nursing strong pressure is being exerted to permit no one to assume any position of responsibility or supervision without at least a Bachelor's degree.[35] With these examples in mind, it seems absolutely obvious that the best training possible should be given those individuals who perform the tests, techniques, and procedures upon which are based the entire medical treatment and future of the patient in the private office, the clinic, or the hospital,—the Medical Technologists.

The American Society of Medical Technologists, taking cognizance of

the acuteness of this problem, recommended a three year prerequisite collegiate program prior to the approved twelve month hospital technical training period required by the Board of Registry of Medical Technologists of the American Society of Clinical Pathologists. This action reiterates the philosophy of the Board of Registry which was first expressed in 1938 in the seventh edition of its booklet, "The Registry of Medical Technologists of the American Society of Clinical Pathologists." It stated, "Schools for training the technologists are encouraged to become affiliated with universities and colleges so that there may be a coordination of the preliminary college studies with those pursued in the training school proper.[43] Universities with adequate hospital facilities are being stimulated to open courses leading to the degree of Bachelor of Science in Medical Technology, the last year to be spent in actual practice in hospital laboratory procedures under the guidance of clinical pathologists."

We might well now consider what type of collegiate program of three or more years should be offered the prospective student interested in the science of Medical Technology. Most colleges or universities offer a three year basic program which include the minimum requirements of the Registry, and allow in most cases the acquisition of a Bachelor's degree at the end of the senior year's hospital technical training. A few institutions have attempted to dignify the degree of Bachelor of Science in Medical Technology by offering courses dealing specifically with Medical Technology as campus courses prior to the senior clinical year. This type of program is needed at the present time, when the complexity of the medical sciences requires the best trained candidates for Approved Hospital Training Schools of Medical Technology. In order to properly approach the problem and benefit the potential Medical Technologist during the hospital technical training program, it is recommended that the college training schools offer a major in Medical Technology during the three year college course of study recommended by the Board of Schools of Medical Technologists, the Board of Registry of Medical Technologists, and the American Society of Clinical Pathologists. This would allow the student to learn his profession and develop his techniques prior to his practical technical training days in the hospital school.

Medical Technology As a Collegiate Program

FRESHMAN YEAR

First Semester		Second Semester	
Course	Sem. hrs.	Course	Sem. hrs.
General Chemistry	4	General Chemistry	4
General Botany (incl. Genetics)	4	General Zoology	4
English Composition	3	English Composition	3
Mathematics	3	Philosophy	3
Logic	3	Hematology & Blood Banking	4
Introductory Medical Technology	1		
	Sem. hrs. 18		Sem. hrs. 18

SUMMER SESSION

Medical Technology Physiology	4 sem. hrs.
Mammalian Anatomy	4 sem. hrs.
	8 sem. hrs.

SOPHOMORE YEAR

Course	Sem. hrs	Course	Sem. hrs.
General Histology	4	Inorganic Quantitative Analysis	4
Survey of English Poetry and Prose	3	Survey of English Poetry and Prose	3
Philosophy	3	Psychology	3
Modern Foreign Language	3	Modern Foreign Language	3
Urinalysis	4	Gastric and Fecal Analyses (Parasitology)	4
	Sem. hrs. 17		Sem. hrs. 17

JUNIOR YEAR

Course	Sem. hrs.	Course	Sem. hrs.
Organic Chemistry	4	Clinical Chemistry (Biochemistry)	
General Bacteriology	4	for Medical Techlologists)	3
General Ethics	3	Animal Microtechniques	4
Modern Foreign Language	3	Individual and Social Ethics	3
Advanced Hematology		Modern Foreign Language	3
Serology, Immunology	4	Clinical Medical Technology (Incl. Instrumentation, Mycology)	4
	Sem. hrs. 18		Sem. hrs. 17

SENIOR CLINICAL YEAR

Hospital Technical Training 8 Sem. hrs. Hospital Technical Training 8 Sem. hrs.

Loyola University started its Department of Medical Technology in 1935. It offers a full four year program in Medical Technology. The above curriculum for this course will be discussed in this paper.

It will be noted that this degree program which has been in operation for over twenty years includes practically every recommendation listed as desirable by the Directors of Approved Schools of Medical Technology in the previously mentioned survey by the American Society of Medical Technologists. The Medical Technology subjects listed in the above curriculum are courses of collegiate level consisting of lectures and laboratory sessions. This collegiate approach permits the student to gain an excellent background in the theory, procedures, and techniques in Medical Technology prior to the start of his prescribed year of hospital technical training.

The Bachelor of Science in Medical Technology program is a worthy addition to the family of academic degrees of any college or university. This degree program, if completed, permits the recipient to enter a most select group of medical scientists. If he does not complete the course of his original matriculation, the foundation which he receives will give him basic courses which he may use in many other fields. For your information, and perhaps guidance of future young people tentatively interested in the Science of Medical Technology, the results of a survey of the careers of some eight hundred people who started in Medical Technology is presented here.

Since the opening of the Loyola University Department of Medical Technology in 1935, its graduates have left their imprint on the community.[1] As of June 1959 the Department has graduated three hundred and sixty Medical Technologists. Those students who enrolled in but did not complete the B.S. in M.T. program at Loyola University have, in many instances, entered careers which have been most noteworthy and have reached into most of the other academic fields.

Several former Medical Technology students have entered the religious life or medical missionary field. Other students of Medical Technology have entered many of the Baccalaureate fields with outstanding success. These individuals have been awarded degrees in the following fields:—B.S. in M.T. (other than Loyola); B.S. in Chemistry; B.S. in Biology; B.S. in Social Sciences; B.S. in Education; B.S. in Physical Education; B.S. in Music; B.S. in Nursing; B.S. in Home Economics; B.B.A.; Ph.B.; and A.B. In addition, a few former students have progressed to the higher education degrees of Master of Arts, Master of Science, Master of Education, and Master of Nursing Education. Other fields in which the former Medical Technologists are active include those of the airline hostess, x-ray technologist, pharmaceutical representative, meteorologist, U.S. State Department, and the armed services.

The Loyola University graduate Medical Technologists, in addition to routine positions in hospitals, clinics, offices of private physicians, research laboratories, and the teaching field, have also brought additional fame to themselves, their community, and the University by their post-university activities. This group includes Doctors of Medicine, Doctors of Dental Surgery, Doctors of Philosophy, numerous and various Masters Degrees, and Lawyers.

We might at this time ask ourselves—why have university trained Medical Technologists had such a far reaching and worthy effect on their communities? I believe that the answer lies in their overall training. A graduate Medical Technologist has received excellent cultural training, especially in sociological and philosophical courses given in conjunction with his scientific training. The Dean of one of our Medical Schools appropriately characterized the Medical Technologist as a "Sentinel of Truth."[7] He stated that the Medical Technologist must be a person who lives on a high level of moral integrity, and is motivated in his activities by all the virtues and motives which activate the physician in his love for suffering humanity.

When one reviews in his mind how great a privilege it is to serve in the field of Medical Technology he realizes that proper education in this field is most essential. With a brief review of the situation, one may feel confident that the education of the student and his hospital technical training is uppermost in the minds of educators and pathologists associated with this problem. However, it probably should be stated at this time, that the education in this field should not stop with graduation and/or registration as a Medical Technologist.

Education in the field can be accomplished in many ways. One of the most important is to maintain membership in the local, state and national societies of Medical Technology. In this regard some of the advantages would include the privilege of attending Conventions, Seminars, Workshops and Refresher Courses. To keep abreast of the new developments in the field even though personal problems and activities might not allow the Medical Technologist to be as active as he might desire. Also membership assures the individual of the Journal of the American Society of Medical Technologists, periodic newsletters and association with people still active in the field. Membership in societies also leads to benefits to the profession as a whole. One of the fairly

recent benefits is the recognition of the field of Medical Technology to the extent that commissions are now offered Medical Technologists in the Armed Forces. This was brought about by the combined activities of the American Society of Clinical Pathologists, the American Society of Medical Technologists, the Board of Registry of Medical Technologists, the Board of Schools of Medical Technologists and interested pathologists and educators.

Medical Technologists are probably aware of the recent controversy which occurred with reference to the allocation of professional rating in government Hospitals for Medical Technologists. An excerpt from a letter written by an officer of the U.S. Civil Service Commission is given here. He stated—"We have recently completed a nation-wide restudy of medical technician positions in the government service. In the course of this study particular attention was given to the propriety of recognition of the technical work performed in clinical laboratories as being professional in nature. A thorough analysis and evaluation was made of the information and material accumulated on a large sample of various types of medical technician positions, including information on the qualifications of incumbents. As a result we have concluded that the work of these positions, as they operate in the Federal service, requires for its performance something less in training, experience, and/or education than that required for a college degree or its equivalent. Accordingly, these positions are found to be properly classifiable as nonprofessional.

"Before reaching the above conclusion, careful consideration was given to such factors as (a) technical responsibility for reporting results to pathologists and/or other professional employees; (b) various regimens through which training, experience, and education have been acquired by medical technicians in many laboratories; and (c) comparability of technical duties and responsibilities and qualifications required for the performance of Medical Technician work with those of other positions in hospitals and clinics.

"However, our study has resulted in a tentative conclusion that the technological advances in medical technician work over the last several years justify a somewhat higher grade structure for this occupation. We are distributing a tentative draft of revised position-classification standards to Federal Agencies and interested organizations for their comment. We hope to forward the final standards to the Government Printing Office by February 1956; for printing and distribution to the employing agencies."

Needless to say this attitude created quite a stir. Organizations interested in the welfare of Medical Technologists again took concerted action in this matter, and after considerable effort and negotiation, the Government agreed to reclassify Federal Medical Technologists as "professional" workers. This is brought to your attention to show that membership in organizations will not only benefit you educationally, but also in unity will give you strength for your own protection.

One additional thought in the educational picture concerns what is wrong with modern education. A commentator, in a recent television appearance, stated that the trouble with modern education is that there

are no real standards and that there is a definite lack of public appreciation for the value of a diploma or a certificate of educational accomplishment. Therefore, in conclusion Medical Technologists should accept the responsibility of raising their educational standards and keeping their profession at a high level of progressive service, so that all their efforts will always have the full appreciation and respect of the public, and their coworkers in the field of Medical Sciences.

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Recent Deaths

— 1959 —

Aretas Evans Bahn, Illinois

Mary Ellen Bittner, Montana

Lois E. Downs, Ohio

Thomas L. Herndon, Indiana

Mrs. Madeline Norris, New Mexico

Sr. M. Irmena Olds, Missouri

Sr. M. Celeste Frances Reilly, N. J.

Frieda M. Oltmanns, Nebraska

Charlotte L. Taw, Pennsylvania

Sr. M. Edwardine Tiell, N. D.

Alice Snyder, Maryland

ELEVATION OF BLOOD UREA NITROGEN FROM THE ADMINISTRATION OF CHLORAL HYPNOTICS*

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Introduction

The interference of chloral hydrate and chlorobutanol on the blood urea nitrogen (BUN) determination by direct Nesslerization has not been reported. The object of this paper is to show the effect of various concentrations of chloral hydrate and chlorobutanol on the BUN determination. Various amounts of chloral hydrate were administered intravenously to mongrel dogs and orally to human beings, and the BUN concentration of the blood determined.

Methods and Experimental

Fasting blood samples were obtained from nine patients, and chloral hydrate was added in 1.0 ml. quantities to give concentrations of 0.5, 1, 2, 3, 4, 5 and 6 gms. %. Folin-Wu filtrates[1] were prepared and the BUN determined by direct Nesslerization using the following method: Reagents:

1. Urease tablets were obtained from Hynson, Westcott and Dunning, Inc., Baltimore, Maryland.

2. Ammonium Sulfate Standard: Stock: 1 cc. = 1 mg. nitrogen (4.7165 gms. dried NH_2SO_4 to 1 liter). Working: 1 cc. = 0.01 mg. of nitrogen. Dilute 1 cc. stock to 100 ml. with distilled water.

3. Nessler's Solution: Made up according to the Folin Wu method in Hawk, Oser and Summerson:[3] Folin Wu filtrate as follows:

Water	7 cc.
Whole blood	1 cc.
1 urease tablet	

Mix immediately and incubate at 50° C for 15 minutes mixing 2-3 times during incubation. Remove and add:

2/3 N H_2SO_4	1 cc.
10% Sodium Tungstate	1 cc.

Shake and filter. Into Colorimeter tubes place:

	Blank	Standard	Unknown
Distilled Water	9.0 cc.	6.0 cc.	8.0 cc.
Standard		3.0	
Filtrate			1.0

Mix and add 1 cc. of Nessler's Reagent to each tube. Mix and read in exactly 10 minutes against No. 54 filter.

The percent increase over control BUN following oral administration of 3.0 and 3.5 gms. chloral hydrate respectively to two female patients was determined. Fasting samples of blood were taken 0, 40, 60, 90, 120, 180 and 300 minutes later and the BUN determined.[2]

Three-hundred mg./kg. chloral hydrate were administered intravenously to three male, mongrel dogs and one received 400 mg./kg. Fasting blood samples were taken at 15, 25, 35, 45, 55 and 60 minutes later and the percent increase of BUN over controls determined.[2]

*Read by title 27th Annual Convention of ASMT, Phoenix, Arizona, June 1959. Received for publication February 1959.

The effect of chlorobutanol on the determination of BUN by direct Nesslerization was measured. Fasting blood samples were obtained from three patients and chlorobutanol was added in 1.0 ml. quantities to give concentrations of 100, 200, 300 and 400 mg. %, Folin-Wu filtrate[1] prepared and the BUN determined.[2]

Two-hundred mg./kg. chlorobutanol was administered to three male, mongrel dogs and fasting blood samples taken 0, 40, 85, 120, 180 and 200 minutes later and the BUN determined.[2]

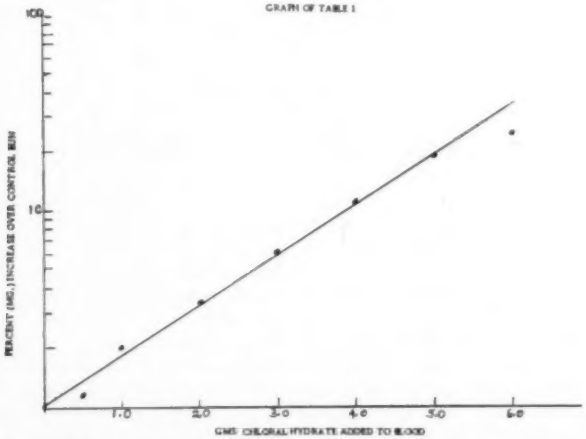
Results and Discussion

The results in Table 1 show the increase in the BUN following the addition of various amounts of chloral hydrate to nine different fasting blood samples. The BUN is exponentially proportional with increasing amounts of the sedative.

TABLE 1

The Effect of Chloral Hydrate on the Determination of Blood Urea Nitrogen by Direct Nesslerization

	Percent (Mg.) Increase Over Control Percent (Gm.) Chloral Hydrate Added to Blood						
0	0.5	1	2	3	4	5	6
BUN mg. %							
18.8	1.5	...	...	...	...	...	...
16.3	...	1.4	2.3	7.5	9.6	13.7	26.4
18.3	...	...	2.9	...	...	...	20.8
14.7	...	1.8	4.1	8.3	12.1	22.4	30.5
20.0	0.5	2.2	2.7	3.6	10.0	17.2	20.9
23.4	...	...	4.8	...	...	...	...
14.1	1.9	2.8	3.7	6.7	10.2	20.7	25.0
22.3	0.9	2.0	2.9	4.2	11.1	19.4	24.6
15.2	0.9	2.2	3.0	6.9	13.9	...	...
Average	1.14 ± .49	2.07 ± .42	3.30 ± .77	6.20 ± 1.72	11.15 ± 3.56	18.68 ± 6.74	24.70 ± 8.15



Mean values of the effect of chloral hydrate on the determination of Blood Urea Nitrogen by Direct Nesslerization.

Three and three one-half gms. of chloral hydrate were orally administered to two patients (Table 2). The maximum increase of BUN concentration over the control was 90 minutes following administration of chloral hydrate with a rise of 3.7 and 5.7 mg. % respectively.

TABLE 2

Percent (MG.) Increase Over Control Blood Urea Nitrogen Following Oral Administration of Chloral Hydrate to Patients

	Time (Min.)						
DOSE	0	40	60	90	120	180	300
gms.	BUN mg. %						
3.0	10.9	2.4	2.4	3.7	2.4	4.1	....
3.5	9.1	3.8	6.3	5.7	...	4.8	4.4

Table 3 shows the results from intravenous administration of chloral hydrate to mongrel dogs. The increase in BUN concentration over the control reached a maximum between 25 to 45 minutes following the administration of chloral hydrate.

TABLE 3

Percent (MG.) Increase Over Control Blood Urea Nitrogen Following Intravenous Administration of Chloral Hydrate in Dogs

			300 mg./kg.				
			Time (Min.)				
Body Wt.	0	15	25	35	45	55	60
kg	BUN mg. %						
11.0	27.7	5.6	...	...	3.7	...	1.7
12.0	9.8	3.9	8.2	....	6.4	1.3	...
10.5	14.6	2.0	...	4.8	...	3.2	...
			400 mg./kg.				
11.5	22.9	....	3.4	...	3.9	...	1.5

The results obtained when chlorobutanol was added to four different fasting blood samples are shown in Table 4. The BUN increased as the dosages became greater in concentration.

TABLE 4

The Effect of Chlorobutanol on the Determination of Blood Urea Nitrogen by Direct Nesslerization

Percent (Mg.) Increase Over Control				
	Percent (Mg.) Chlorobutanol Added to Blood			
0	100	200	300	400
BUN mg. %				
29.7	...	...	...	13.7
16.2	1.5	3.6	6.2	10.4
18.9	1.3	3.6	...	....
18.1	0.9	...	3.7	8.1

Table 5 shows the increase of the BUN following intravenous administration of chlorobutanol to three dogs. This hypnotic has a longer sedative effect on the animal than chloral hydrate.[4] The maximum increase in BUN concentration over the control was obtained within 3 hours.

TABLE 5

Percent (MG.) Increase Over Control Blood Urea Nitrogen Following Intravenous Administration of Chlorobutanol in the Dog

	209 mg./kg.					
	Time (Min.)					
Body Wt.	0	40	85	120	180	200
kg.	BUN mg. %					
10.5	12.1	4.8	5.7	8.7	8.0	...
9.5	14.5	2.6	...	6.9	9.9	7.7
12.5	12.8	1.7	0.4	2.2	2.2	...

If blood samples are taken for BUN determination by direct Nesslerization following administration of chloral hydrate and chlorobutanol, there will result a false positive rise in the BUN concentration.

Conclusion

The effects of the chloral hydrate and chlorobutanol on the determination of blood urea nitrogen (BUN) by direct Nesslerization has been determined. There is a proportional increase in BUN concentration with increasing amounts of the hypnotic agents added to blood. The administration of chloral hydrate to human beings and mongrel dogs and chlorobutanol to dogs results in a false positive rise in the concentration of BUN by direct Nesslerization.

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ON THE ACHIEVEMENT OF OBJECTIVES*
(Methodology of Accomplishment)

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Clearly understood, constructive objectives are necessary to people and organizations. Lack of them brings about a state of aimlessness or weakness which may precede confusion, frustration, defeat, and all the resultant unhappiness. What threatens us and our purposes constantly as individuals and groups is confusion, disintegration, subversion.

It is axiomatic for achievement that you must know clearly what you want to achieve. Accordingly, goals have important functions to perform for the person or the group. These are: (a) to unify or integrate the parts, (b) to mobilize all the resources and to safeguard against dissipation of energies, (c) to give meaning, purpose, direction, (d) to give a sense of time perspective.

Objectives may be variously classified or characterized when they exist, as vague or unclear, unrealistic, unattainable, morally wrong, etc. It is, however, more useful to us to note that there are goals which are inherent or natural to our situation (a) as human beings, (b) as professional people, ignorance or neglect of which might conceivably constitute delinquency.

What are these aims?

As human beings (in contrast to the brute or beast) is it not a "natural" goal, in the sense of being "good", inherently "right", to be creative, integrative, constructive? And does it not follow that the aims natural and necessary to the ultimate goal of creativeness or constructiveness are the cultivation of the virtues of kindness, patience, freedom from envy, jealousy, spite, resentment, anger, revenge, pride, prejudice; the freeing of ourselves from the cynicisms, pessimisms and doubts which block or destroy creativity (because they are anti-knowledge and anti-understanding)? Are not the goals likewise those working towards the extension of Human Dignity and Reverence for Life? Are these not "natural", "inherent", "right", "necessary" aims for human beings without clear notion of which one cannot do or be his best?

Now what are such characteristic aims for professional people? The nature of a profession has been described by Dr. Vannevar Bush,** and accordingly professional people are to: 1. know clearly what a profession is; 2. attain the primary characteristics of professional people, namely: "to administer unto people—and serve them with dignity, devotion and skill"; 3. occupy themselves with such intermediate goals: the problem of recognition, attainment and maintenance of sufficient dignity, devotion and skill, as to properly serve as the professional aims require.

Knowing or recognizing our objectives, then how are they to be accomplished?

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**In Dr. Leonard A. Scheele's address, *Year of the Turning Point:* 1958, before the 26th annual convention of the ASMT, Milwaukee, Wis., June, 1958. See Amer. J. Med. Tech., July-Aug. 1958.

It should be known that there is a general methodology or technology of accomplishment. Visit the local library. Some authorities believe it should be studied as a separate study. Basic to it is: 1. The clear, well expressed idea of the objective; 2. a moving desire to accomplish that objective; 3. a realistic plan to accomplish the goal; 4. action on the plan; 5. re-examination of the plan and revision subsequent to action; 6. ways and means of recovery from accident, upset, disaster, delay, failure, fatigue, exhaustion; 7. self-supervision all the way to accomplishment.

The plan is central and critical. It must be complete, all-inclusive. Plan is the means whereby man exercises control over events which would otherwise have control over him. Control is the function of plan. It is also the function of self-supervision. To plan effectively one needs to (a) 1. make adequate observations of the problem; 2. consult experiences of experts; 3. read the literature concerned: (b) 1. express the ways and means of reaching your goal based on preceding observations; 2. express intermediate ways and means which will get you to your goal; the setting of intermediate or day-to-day goals; 3. ask certain basic questions to insure thoroughness of analysis and planning; 4. look for the seemingly irrelevant factor which may become the key to the solution. *Questions which are not asked cannot be answered.*

Achievement of objectives is fundamentally a matter of problem solving. This has its own general methodology, the characteristics of which have been here briefly outlined. But first and all the way to the final achievement is the matter of desire. No human movement toward goals occurs without sufficient desire to act, and act, and act according to goal and plan until achieved. Desire causes the goal to be, then to be clearly expressed, then to be planned for, then to be acted upon. Desire *self-supervises* the process all the way to the achievement of the goal.

When desire is absent or weak, then the objective is obvious; to inspire it. This is a matter for study, thought, plan, and action according to a definite methodology and close, thoughtful *self-supervision* through that methodology.

Whatever the goal, be it the attainment of a specific skill, a body of knowledge, the conclusions of a scientific investigation, the attainment of professional dignity and devotion; or peace of mind, contentment, happiness; its achievement is the product of motivating desire toward a clearly expressed goal by means of a realistic plan, closely *self-supervised.*

THE USE OF LECTINS IN BLOOD BANKING*

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The discovery of plant extracts with definite specificities has made two satisfactory antisera available for blood bank work:

1. Dolichos biflorous for subgrouping A and AB blood.
2. Ulex europeus for differentiating secretors of all types from non-secretors, especially group O.

Before these lectins became available, absorbed B serum was used exclusively for subgrouping groups A and AB bloods; and for the separation of group O secretors from non-secretors, anti-O or H serum was used. The sources of supply of this latter serum were limited to: 1. The rare A_1B person with anti-O in his blood. 2. The serum of a goat immunized with Shiga's bacilli. 3. Eel's blood which sometimes contains anti-H, a source of small and unreliable yield.

Plant extracts are variously referred to in the literature as phytagglutinins, plant agglutinins, hemagglutinins and lectins. The term "agglutinin" as used in blood banking refers to an antibody formed in response to stimulation by an antigen. Plant extracts are not antibodies. They are part of the cellular structure of the plant. They are protein or mucoprotein in nature, and may have the specific property of differential agglutination of human or animal erythrocytes. For this reason Boyd[5] prefers to call them "lectins" from the Latin "legere" to choose or pick out.

The ability of lectins to agglutinate human and animal blood was noticed many years ago, and has had wide and intensive study. In 1888 Stillmark[17] found that erythrocytes of certain animals were agglutinated by extracts of ricin. Landsteiner in 1925[9] pointed out that some plant extracts demonstrated various types of protein specifity. So rapid was the advance in this field, that by 1955 more than 1000 plant species had been studied. Makela[11,12] in 1957 published a comprehensive and detailed monograph on plant agglutinins. He found that 69 of the 743 plant species, mostly Leguminaceae, which he studied agglutinated human erythrocytes selectively. For his specificity tests he chose panels of human cells which included the blood factors A, B, O, M, N, P, S, s, Le^a and Le^b. He used also the cells of various animals; cow, sheep, goat, rabbit, chicken, and guinea pig. He found plant extracts which were specific for groups A, A and B, B, and types H and N. Some of these extracts reacted better in albumin or polyvinylpyrolidine like univalent antibodies, others were enhanced by the use of papainized cells. But the majority worked best in a solution of normal [isotonic] saline.

Bird and Levine[2,3,4,10] each reported on the interrelationship of plant agglutinins and the red blood cells of various animal species. In 1957 Bachrach[1] announced that he had found in Israel plants with specific hemagglutinins. McNeil[13,14,15] used an anti-H lectin in his work on "aberrant secretors." In 1958 Wiener[19] stated that the use of the extract of Ulex europeus in the grouping of dried blood stains has "provided the internal control needed for the reliable grouping of dried blood stains

*2nd Award Blood Banking, Scientific Products Foundation, 1959. Read before the 27th Annual Convention of ASMT, Phoenix, Arizona, June 1959.

for individuals of group O and A_1B." In the same year [1958] Van Arsdal[18] found Ulex of great value in determining the secretor status of large groups of humans. He studied 3,114 persons and found that 24%, the expected number, were non-secretors.

For the preparation of extracts of both Dolichos biflorus and Ulex europeus the method recommended by Boyd[6,7,8] is used. It is simple, and gives a uniform and reliable product.

I. Method for the Preparation of Both Lectin Extracts: Dolichos and Ulex:

A. The seeds are ground to a meal in a mill. [A small pepper mill will do.]
B. To each gram of meal 10 cc of normal saline is added.
C. The mixture is then agitated for one hour on a Kahn shaker, and centrifuged in large test tubes at high speed for five minutes. The clear, yellowish supernatant contains the lectin.
D. This is poured off, and may be used "as is" or filtered through a Seitz filter.
E. The filtrate is distributed in 2 cc vials and frozen for storage. [Lectins do not lose their potency with repeated freezings and thawings.]
F. For immediate use a one or two cc vial can be kept in the refrigerator at 4° C. without loss of potency.

II. Technic of the Tests:

A. For the subgrouping of A_1 and A_1B blood with Dolichos biflorus extract, tests can be performed in tubes or on slides.
B. For the identification of A_2 and A_2B and O blood with Ulex europeus extract, tests can be performed in the same manner. (This procedure not recommended for routine use.)
   1. Tube Test:
      a. Use an 8.5 x 75 mm tube for each test plus one tube for a positive, and one for a negative control.
      b. To each tube add one drop of the extract.
      c. Add a stick of cells* from the clot of each blood to be tested.
      d. For the positive control for Dolichos use A_1 cells, and for the negative control use A_2 cells.
      e. Centrifuge for one minute and read macroscopically.
      f. In positive tests the cells agglutinate in a solid mass which cannot be shaken apart, while in negatives the cells shake out easily.
   2. Slide Test:
      a. Place a drop of the whole blood to be tested on a slide.
      b. Also place a drop of the negative and a drop of the positive controls on the same slide.
      c. Add a drop of the extract to each.
      d. Mix with an applicator stick and place on the light-box to read.

* A stick of cells is made by inserting a wooden applicator stick into the clot. When the stick is withdrawn red cells adhere to its surface. Agitation of the stick in the serum loosens the cells.

e. There is immediate, strong agglutination in the positives, whereas the negatives remain evenly smooth and unagglutinated.
f. Final readings should be made at the end of two minutes.

C. For Demonstrating Secretors and Non-secretors in Saliva with Ulex.

1. Collection of Saliva.
a. About 3 cc of saliva is collected in a test tube.
b. Secretor and non-secretor controls of the types to be tested are collected at the same time.
c. They are placed in a boiling water bath for 20 minutes to destroy enzymes and kill bacteria.
d. They are then centrifuged at high speed for 5 minutes and the supernatant poured off into tubes.
e. The supernatant can be kept in the refrigerator at 4° C. if the tests are to be done in a day or two, or frozen for longer storage.

2. Technic of the Test.
a. To 0.1 cc of saliva supernatant add 0.1 cc of Ulex extract.
b. Mix by shaking.
c. Let stand at room temperature [25° C.] for 30 minutes.
d. Transfer one drop of the mixture to another tube and add one drop of a 5% saline suspension of washed group O cells.
e. Set up a control tube of Ulex and group O cells, another of group O cells in saline, and secretor and non-secretor controls for the series.
f. Centrifuge and read tubes immediately; or allow to stand at room temperature for an hour and read without centrifuging.

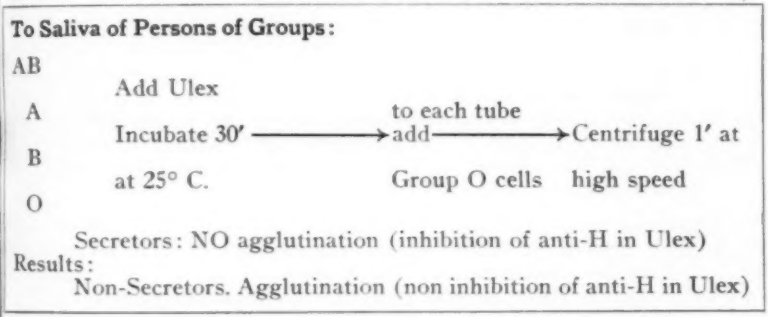

Figure 1
Diagram of the Simplified Saliva Test Using Ulex europeus to Separate Secretors from Non-secretors.

III. Results:

A. With Dolichos biflorus:

1. Dolichos biflorus agglutinated A_1 and A_1B cells, but not A_2, A_2B, B or O cells.

2. In more than 1000 tests with this lectin no false positives or negatives were found.

B. With Ulex europeus:

1. Stick tests were done on 225 blood samples.

Type	Totals	Positive	Negative
A_1B	9	0	9
A_2B	9	8	1
A_1	66	14	52
A_2	10	10	0
B	30	18	12
O	101	101	0
Totals	225	151	74

2. Ulex agglutinated 100% of A_2 and group O cells.
3. Of the 66 A_1 samples 14 were agglutinated and 52 were not.
4. Eighteen of the 30 group B bloods were positive and 12 were negative.
5. Nine A_2B bloods were tested. Eight were positive and one negative.

No explanation for the behavior of Ulex with A_1 and B blood can be offered at this time. The total number of specimens tested is too small, and the percentages derived too far removed from the expected frequency for these results to indicate AO and BO heterozygosity. The A_2B blood, negative with Ulex, was checked with several subgrouping antisera and corroborated as an A_2B. Race and Sanger[16] gave an instance of the failure of an anti-H lectin to agglutinate A_2B cells. They conclude that this phenomenon is a "curious" reaction.

C. With Ulex europeus for demonstrating secretors and non-secretors.

1. In secretors the group O cells are not agglutinated.
2. In non-secretors the group O cells are agglutinated.
3. Because secretors of A, B and O substance also secrete H substance, the anti-H in Ulex is neutralized by the H substance in the saliva of secretors and none is left to agglutinate the group O cells added. Therefore they are not agglutinated. With non-secretors, there is no H substance present in the saliva. The anti-H is not neutralized and agglutinates the O cells.

IV. Conclusions:

A. Lectins have the following advantages:

1. They are cheap and easy to prepare.
2. They can be kept indefinitely if frozen, and thawed to be used as needed. Innumerable thawings and refreezings do not impair the potency of the lectin if contamination is avoided.
3. The tests are rapid, easy to perform, and give strong, clear, immediate readings.
4. The easy availability of lectins makes it possible for even the smallest blood bank to perform two more services:
   a. The subgrouping of A and AB blood.
   b. The separation of secretors from non-secretors of all types, especially group O.

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THE EFFECT OF RADIOGRAPHIC CONTRAST MEDIA ON URINALYSIS†

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Introduction

As medical science progresses, new diagnostic and therapeutic preparations are introduced, and as a result, some diagnostic tests which formerly were considered reliable are found to give atypical or misleading findings. This necessitates alertness on the part of the Medical Technologist to recognize these deviations.

In our laboratory, Medical Technologists working in the urinalysis department in recent years have encountered perplexing situations in analysis of urine specimens of patients who had received intravenous injections of radiopaque medium for X-ray purposes.

This paper is an attempt to help clarify the situation by showing the effect of one medium, Hypaque sodium* on semi-quantitative urine examinations.

A survey of recent literature did not reveal that there have been any publications on this subject. However, literature accompanying the medium states that it does not interfere with the heat and acetic acid test for albumin.

Materials and Methods

Twenty-three patients on whom intravenous urograms were performed by the department of radiology in Beckley Memorial Hospital, were used in this study. One urine specimen was collected prior to the intravenous injection and a second was obtained one-half hour afterwards.

The analysis of each specimen was performed within nine hours of collection. It must be stated here that certain tests used in this study should be determined within a very short time to be valid, but since the pertinent data desired in this study was of a comparative nature, we felt that we could ignore the time factor.

The analyses were conducted as follows:

(1) The specific gravity of each base line sample was estimated with a Vogel type urinometer, calibrated for 25° C; the post-intravenous samples were estimated with a Squibb type urinometer, calibrated for 16° C. Both readings were corrected for room temperature (usually 28-29° C.).

(2) The albumin was estimated with the heat and acetic acid test, 20 per cent sulfosalicylic acid, nitric acid ring test, and "Uristix"[1] test.

(3) The pH was obtained by "Hydrion" paper.[2]

(4) The urobilinogen was estimated by Ehrlich's qualitative method. If a yellow precipitate was obtained, a test for sulfa was done.

*Hypaque sodium is a trade name for sodium 3,5 diacetoamide 2,4,6, triiodobenzoate and is supplied by Winthrop Laboratories.

[1] Uristix is the trade name for an albumin paper stick test and is supplied by Ames.

[2] Hydrion paper is a qualitative pH indicator in paper stick form supplied by Ames.

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(5) The sugar was estimated by Benedict's qualitative method for sugar.

(6) A microscopic examination was performed.

All equipment, reagents, and methods for the above-mentioned tests can be found listed in most standard text books of clinical laboratory diagnoses.

The interpretations of results were made by comparing the results of the two examinations using the specimen collected prior to the injection of Hypaque as the base line sample.

Results

The specific gravity showed an increase in the post-intravenous samples in all but two cases. These changes varied between .001 and .059 with fifteen of the samples showing more than .026 change.

The administration of radiopaque material had no effect on the reading of the albumin by the heat and acetic acid and by the "Uristix" test; however, the nitric acid ring test and 20 per cent sulfosalicylic acid yielded strong reactions in several of the post-intravenous specimens.

The pH as estimated by pHydrion paper showed the same readings on the post-intravenous specimens as on their base line samples.

In Ehrlich's test for urobilinogen, fifteen of the post-intravenous specimens gave a heavy yellow precipitate in contrast to their base line samples, which showed normal reactions. One other sample gave a slight cloudiness in the post-intravenous sample. The sulfa test was negative in all these cases.

The results of the specific gravity changes, the urobilinogen reaction, and the albumin as determined by 20 per cent sulfosalicylic acid and nitric acid of the post-intravenous samples are combined in Table I.

TABLE I

Cases	Spec. Gravity Change	Albumin	Urobilinogen
6	.006 (0—.012)	Neg.	Neg.
1	.013	±	Cloudy
1	.015	4 plus	Heavy Precipitate
15	.039 (.026—.059)	4 plus	Heavy Precipitate

This table shows the significant results obtained from the analysis of the urine from twenty-three patients after intravenous injection of Hypaque. In seventeen of the cases, there is a parallel relationship between the increase in specific gravity, and the misleading findings of the qualitative albumin tests using 20 percent sulfosalicylic acid and nitric acid, and of the qualitative urobilinogen tests using Ehrlich's reagent.

The sugar determination by Benedict's qualitative method gave the same readings in the post-intravenous samples as in their respective base line samples.

The microscopic examinations showed the same results in the post-intravenous samples as in their respective base line samples.

Comments

In fifteen of the twenty-eight cases studied, a consistent combination of abnormal results was obtained. The specific gravity increased markedly (with an average of .039 points), the 20 per cent sulfosalicylic acid and the nitric acid ring test for albumin gave four plus readings, and the reaction with Ehrlich's test produced a heavy yellow precipitate in all these post-intravenous specimens.

The precipitate formed in Ehrlich's test and in the nitric acid ring test formed instantly, but the precipitate formed in the 20 per cent sulfosalicylic acid test sometimes required 30-90 seconds.

Since the heat and acetic acid test readings in these fifteen post-intravenous specimens remained the same as their respective base line, it is the test of choice to use on urine specimens suspected of containing Hypaque sodium.

There was no formulated study made to determine how many hours after injection of Hypaque sodium one might expect to find the above results, but a few perfunctory tests seem to indicate that the urine findings tend to revert back to those of the base line sample within 6 to 8 hours.

Summary

(1) To determine the effects of a radiographic contrast medium on routine urinalysis, urine examinations were performed on urine specimens collected from 23 patients 15 to 30 minutes after an intravenous injection with Hypaque sodium. The results were compared with a base line sample.

(2) In 15 of the 23 cases studied, the individual post-intravenous urine samples showed a definite pattern of abnormal findings which were as follows:
   a. Marked increase in specific gravity.
   b. False 4 plus albumin readings with the nitric acid ring test and the 20 per cent sulfosalicylic acid test.
   c. A heavy yellow precipitate in Ehrlich's test for Urobilinogen.

(3) The pH determination using "Hydrion" paper, the glucose determination using Benedict's reagent, the albumin determination employing the heat and acetic acid and "Uristix" test, and the microscopic examination, showed no significant change from the base line results.

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URINALYSIS AND ORAL HYPOGLYCEMIC AGENTS*

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Introduction

Oral hypoglycemic agents have become important in the control of diabetes. Urinalysis is of great value in patients receiving these agents and for this reason it is desirable to know if generally used procedures can be employed with confidence.

This report describes studies carried out to determine the performance of laboratory and commercial tests with urine containing oral hypoglycemic agents or their metabolites.

Experimental

Five oral hypoglycemic agents which are sulfonylurea compounds were used in this study. They are tolbutamide, carbutamide, chloropropamide, metahexamide and methoxy isobutyl sulfathiadiazole. In addition, phenformin, an oral hypoglycemic agent, which is a biguanide compound, was used.

To determine the effect of the presence of the oral hypoglycemic agents or their metabolites in urine on diagnostic testing procedures, two studies were conducted. In one study, urine specimens were collected from three different species of animals which had received large doses of each of the above oral hypoglycemic agents. In the other study, each of the oral hypoglycemic compounds was dissolved in human urine samples in an amount larger than normally would be expected to be present after ingestion of the agent.

Tests Employed and Methods of Testing

The procedures employed are shown in Table I. Some of these are laboratory procedures and some are commercially available tests. The directions for the laboratory procedures employed are those described in recent textbooks of clinical pathology (1) (2), and the directions for the commercial tests used were those supplied by the manufacturer. A total of 23 different urine test procedures was employed.

Studies With Animal Urines

Six of each species, dogs, guinea pigs and rats, were employed. During the study, each of the animals was housed in a separate cage and given food and water *ad libitum*. Immediately after urine collection, all urine specimens were stored under toluene in the refrigerator. From all animals, a 24-hour control urine was collected. At the end of the control period, each of the six hypoglycemic agents was administered to a separate animal of each species. Urine collections were obtained from all animals during 24 and 48-hour periods after drug administration. These urines (a total of 54 samples) were thoroughly examined as to their reactivity with several diagnostic tests.

For administration, the hypoglycemic agents were suspended in 1 per cent carboxymethylcellulose. Phenformin was an exception since it is

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TABLE I

Urine Tests Employed

Urine Sugar
Copper Reduction — Benedict's, CLINITEST
Enzymatic — CLINISTIX, URISTIX, COMBISTIX

Urine Protein
Turbidimetric — BUMINTEST, Nitric Acid Ring, Heat and Acetic Acid, Exton's
Colorimetric — ALBUSTIX, ALBUTEST, URISTIX, COMBISTIX

Urine Ketones
Nitroprusside — ACETEST, Rothera Stratification, KETOSTIX
Ferric Chloride — Gerhardt's

Urine Bilirubin
Oxidation — Watson's Strip Test, Harrison's
Diazotization — ICTOTEST

Urine Urobilinogen
Wallace and Diamond Test

Urine Occult Blood
OCCULTEST

Clinitest, Clinistix, Uristix, Combistix, Bumintest, Acetest, Albustix, Albutest, Ketostix, Ictotest, Occultest are registered trademarks of the Ames Company, Inc., Elkhart, Indiana.

water soluble. All agents were administered by stomach tube and the doses given were much larger than the equivalent doses recommended for human patients (3). Table II shows the amount of each of the compounds administered.

Results were negative on all animal urines using all of the sugar tests listed in Table I. When glucose in quantities ranging from 0.1% to 1% was added to each urine sample, the expected positive results were obtained.

With the exception of Gerhardt's test, all ketone tests listed in Table I were employed on all animal urines. The results of these tests were all negative, and when quantities of acetoacetic acid ranging from 5 to 80 mg.% were added to these urines, the expected positive results were obtained.

TABLE II

Dosage to Animals and Amounts Added to Human Urine

Compound	Dosage to Animals mg./kg. Body Wt.	HUMAN URINE		
		Amount Added mg. %	Amount Dissolved	
			pH 5.5 Urine mg. %	pH 7.5 Urine mg. %
Metahexamide	200	100	77	100
Carbutamide	500	500	68	500
Methoxy Isobutyl Sulfathiadiazole	200	300	13.5	300
Chlorpropamide	200	500	46	500
Tolbutamide	1000	1500	5.5	539
Phenformin	200 (rat) 100 (dog) 50 (guinea pig)	200	200	200

All protein tests listed in Table I were employed on the animal urines with the exception of COMBISTIX, which was not at the time developed in its final form. All of the colorimetric tests gave negative results with all of the animal urines. The turbidimetric tests gave some false positive reactions with the animal urines as indicated in Table III. It is notable that BUMINTEST did not give any false positive reactions with any of the animal urines. When quantities of bovine serum albumin, ranging from 15 to 100 mg.% were added to the animal urines, positive results were obtained from all urines.

TABLE III

List of Abnormal Results on Animal Urines

Test	Drug Administered	Abnormal Results
Nitric Acid Ring (protein)	Metahexamide	Trace Pos. on Negative Urine from Dog
Nitric Acid Ring (protein)	Carbutamide	2+ Pos. on Negative Urine from Dog.
Nitric Acid Ring (protein)	Methoxy Isobutyl Sulfathiadiazole	Trace Pos. on Negative Urine from Dog.
Heat and Acetic Acid (protein)	Metahexamide	1+ Pos. on Negative Urine from Dog.
Heat and Acetic Acid (protein)	Carbutamide	2+ Pos. on Negative Urine from Dog.
Exton's Sulfosalicylic Acid (protein)	Metahexamide	Trace Pos. on Negative Urine from Dog.
Exton's Sulfosalicylic Acid (protein)	Carbutamide	2+ Pos. on Negative Urine from Dog.
Exton's Sulfosalicylic Acid (protein)	Carbutamide	2+ Pos. on Negative Urine from Guinea Pig.
Exton's Sulfosalicylic Acid (protein)	Methoxy Isobutyl Sulfathiadiazole	Trace Pos. on Negative Urine from Dog.

Urine bilirubin, occult blood and urobilinogen tests as listed in Table I were employed on dog urine samples only. The small animal urines were not available due to the insufficient quantities of urine. The results were negative for bilirubin and urobilinogen on all the urines studied and the expected positive results were obtained for bilirubin and urobilinogen when these substances were added to the urines in sufficient quantities to give positive results. The tests for occult blood all gave anticipated results.

Studies With Human Urines

Two urine specimens were used, one at pH 5.5, specific gravity 1.015 and the other at pH 7.5, specific gravity 1.020. To each of six 30 ml. aliquots of urine at pH 5.5, and six 30 ml. aliquots of urine, at pH 7.5, a different oral hypoglycemic agent was added. The twelve samples and two control samples, one at each pH, were shaken for 5 hours in a water bath at 37° C and filtered at the end of that time, if necessary. The amount of each of the agents added and the amount in solution, after shaking in the water bath, are shown in Table II. The concentration of dissolved compound was determined from the absorbance of these urine samples in the ultraviolet with a DU Spectrophotometer using a previously determined extinction coefficient for each compound.

Each of the human urines with the added oral hypoglycemic agent gave the expected negative result with all of the sugar tests listed in Table I. Correspondingly when glucose was added to the urine all of the tests for sugar became positive.

When the ketone tests shown in Table I were applied to normal human urine containing added oral hypoglycemic agents, the tests were all negative. When acetoacetic acid was added to these urines and they

were re-tested all of the tests gave the expected positive results except Gerhardt's test which gave poor reactions. This test is so insensitive that a positive reaction is seldom obtained until the concentration of acetoacetate approaches 180 mg.%. Urines containing chlorpropamide, tolbutamide, and methoxy isobutyl sulfathiadiazole at pH 7.5 would not give a clearly positive reaction at 180 mg.% acetoacetate because of the presence of a precipitate that failed to disappear upon the addition of more $FeCl_3$.

With the colorimetric protein tests proper reactions occurred with the urines containing added hypoglycemic agents in all instances except with the urines containing phenformin and tolbutamide, the latter at only pH 7.5. The extremely high concentrations of these compounds caused a marked increase in the buffer content of the urines which tended to give trace false positive colorimetric protein tests. This does not appear to be a problem that will be encountered clinically since tolbutamide is not excreted as such in significant amounts in the urine (4), and the clinical dosage of phenformin would not be expected to yield such urinary concentrations. With the turbidimetric tests for protein (nitric acid ring test, heat and acetic acid test, and the sulfasalicylic acid tests, Exton's and BUMINTEST), all of the sulfonylurea compounds tended to produce false positive tests for protein under many of the situations studied. These are shown in Table IV.

When 15-100 mg.% bovine serum albumin was added to the negative urines, all of the tests gave the expected positive reactions except the urine containing methoxy isobutyl sulfathiadiazole. No reaction to ALBUTEST could be obtained for such a urine at pH 7.5 containing 30 mg.% protein. With 100 mg.% protein added to this urine a positive reaction was obtained which did not have as strong a color as is ordinarily observed with this concentration of protein. Since this phenomenon did not occur in the animal urines after oral administration of methoxy isobutyl sulfathiadiazole, and since with decreases in the concentration of the hypoglycemic agent the effect disappears, there is good reason to believe that false negatives would not occur clinically.

As with the animal studies, the human urines did not give false positive reactions to tests listed in Table I for bilirubin, urobilinogen and occult blood. When known amounts of bilirubin, blood and urobilinogen were added, all of the urines gave the expected positive results, except the urobilinogen reaction with the urine containing carbutamide.

Urines were collected from patients receiving chlorpropamide (500 mg. per day) and phenformin (75 mg. per day to 150 mg. per day). Application of all of the tests listed in Table I gave negative results with the exception of some urines which contained glucose or ketone. When either glucose, protein, acetoacetic acid, bilirubin or blood was added to each urine the expected positive results were obtained.

Discussion

From the above results, there is good reason to believe that most of the more commonly used commercial and laboratory diagnostic tests could be used with complete confidence on urines from patients taking oral hypoglycemic agents.

TABLE IV

List of Abnormal Results with Human Urine with Added Hypoglycemic Agents

Test	Drug Added	Abnormal Results
Nitric Acid Ring (protein)	Metahexamide	Trace Pos. on Negative Urine at pH 5.5 and 7.5.
Nitric Acid Ring (protein)	Carbutamide	1+ Pos. on Negative Urine at pH 5.5 and 4+ Pos. at pH 7.5.
Nitric Acid Ring (protein)	Methoxy Isobutyl Sulfathiadiazole	4+ Pos. on Negative Urine at pH 7.5.
Nitric Acid Ring (protein)	Chlorpropamide	2+ Pos. on Negative Urine at pH 5.5 and 4+ Pos. at pH 7.5.
Nitric Acid Ring (protein)	Tolbutamide	1+ Pos. on Negative Urine at pH 5.5 and 4+ Pos. at pH 7.5.
Heat and Acetic Acid (protein)	Methoxy Isobutyl Sulfathiadiazole	4+ Pos. on Negative Urine at pH 7.5
Heat and Acetic Acid (protein)	Tolbutamide	4+ Pos. on Negative Urine at pH 7.5.
Exton's Sulfosalicylic Acid (protein)	Methoxy Isobutyl Sulfathiadiazole	2+ Pos. on Negative Urine at pH 7.5.
Exton's Sulfosalicylic Acid (protein)	Chlorpropamide	Tr Pos. on Negative Urine at pH 5.5 and 2+ Pos. at pH 7.5.
Exton's Sulfosalicylic Acid (protein)	Tolbutamide	2+ Pos. on Negative Urine at pH 7.5.
BUMINTEST (protein)	Methoxy Isobutyl Sulfathiadiazole	2+ Pos. on Negative Urine at pH 7.5.
BUMINTEST (protein)	Chlorpropamide	1+ Pos. on Negative Urine at pH 7.5.
BUMINTEST (protine)	Tolbutamide	2+ Pos. on Negative Urine at pH 7.5.
ALBUTEST (protein)	Methoxy Isobutyl Sulfathiadiazole	Negative on Urine containing 30 mg. % protein at pH 7.5.
COMBISTIX (protein portion)	Tolbutamide	Trace Pos. on Negative Urine at pH 7.5.
COMBISTIX (protein portion)	Phenformin	Trace Pos. on Negative Urine at pH 5.5 and at pH 7.5.
URISTIX (protein portion)	Phenformin	Trace Pos. on Negative Urine at pH 5.5 and at pH 7.5.
ALBUSTIX (protein)	Tolbutamide	Trace Pos. on Negative Urine at pH 7.5.
ALBUSTIX (protein)	Phenformin	Trace Pos. on Negative Urine at pH 5.5 and at pH 7.5.
Gerhardt's (ketone)	Methoxy Isobutyl Sulfathiadiazole	Negative on Urine containing 180 mg. % acetoacetic at pH 7.5.
Gerhardt's (ketone)	Chlorpropamide	Negative on Urine containing 180 mg. % acetoacetic at pH 7.5.
Gerhardt's (ketone)	Tolbutamide	Negative on Urine containing 180 mg. % acetoacetic at pH 7.5.
Wallace and Diamond (urobilinogen)	Carbutamide	Negative on Urine containing urobilinogen at pH 5.5 and pH 7.5.

The major portion of diagnostic testing, where questionable results might be obtained, appears to be where the turbidimetric tests are used for protein determination. This is not too surprising since it is consistent with previous results when isolated oral hypoglycemic agents were studied.[4] However, there is good reason to believe that the colorimetric tests for protein could be used with confidence on all patients' urines when the recommended dosage of these hypoglycemic agents are taken into consideration.

Summary

Studies have been carried out to determine the possible effect of six oral hypoglycemic agents on several common laboratory and commercial urinary diagnostic tests. Urines were collected from rats, guinea pigs, and dogs which had received oral doses of each of the hypoglycemic agents. In addition all of the oral hypoglycemic agents were added to human urines at two different pH values. Observations were also made on urines from patients receiving chlorpropamide and phenformin.

The results indicate that all of the diagnostic tests investigated can be used with confidence during therapy with hypoglycemic agents, with the exception that some of the turbidimetric tests for protein (e.g. sulfosalicylic acid test) may give erroneous results with urines containing sulfonylurea compounds.

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HYDROGEN SULFIDE PRODUCTION BY PSEUDOMONAS AERUGINOSA: A COMPARISON WITH OTHER CHARACTERISTICS*

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Introduction

In 1950 Rozansky, Weber, and Gurevitch (1950) reported their observations of hydrogen sulfide production on Kligler's iron agar by twenty strains of *Pseudomonas aeruginosa*. Prior to this report Ransmeier and Stekol (1942) had reported the production of traces of hydrogen sulfide by this organism from cystine and cysteine, but not from methionine and homocystine, when these amino acids were incorporated in a synthetic medium. Clarke (1953) demonstrated hydrogen sulfide production by two of three strains of Pseudomonas when a peptone base medium was enriched with cysteine, but none of the three strains formed hydrogen sulfide when thiosulfate or sulfite was added to the medium. In the sixteenth edition of *Burrows' Textbook of Microbiology* (Burrows, 1954) and in the eleventh edition of *Zinsser Bacteriology* (Smith, *et al.*, 1957) *P. aeruginosa* is described as a hydrogen sulfide producer; in the former Rozansky, *et al.* (1950) is cited for support, in the latter no reference is given. Despite these reports, in the seventh edition of *Bergey's Manual* (Breed, *et al.*, 1957) no mention is made of hydrogen sulfide production in the description of *P. aeruginosa*.

In order to resolve the discrepancies which exist relative to hydrogen sulfide production of *P. aeruginosa* and to differentiate more readily atypical strains of this organism from similar organisms in clinical specimens, a study was undertaken to compare hydrogen sulfide production with other properties considered more characteristic of this organism (Kovacs, 1956; Gaby and Free, 1953, 1958; Simon, 1956; Gaby and Hadley, 1957; Martineau and Forget, 1958).

Methods

Organisms. Twenty-three strains of bacteria producing the same reaction on triple sugar iron agar slants, i.e., alkaline slant-alkaline butt, were used in this study. These cultures consisted of one stock strain of *P. aeruginosa*, one stock strain of *Alkaligenes faecalis*, three isolates of non-human origin, and eighteen isolates from various human sources such as feces, urine, skin lesions, nasal passages, and sinus drainage.

Media. Since hydrogen sulfide production was the characteristic under study, a medium was devised which was considered more sensitive than commercially available media for the detection of hydrogen sulfide production by *P. aeruginosa*. This medium, gluconate-iron agar, consists of trypticase (BBL), 20 g; calcium gluconate (NBCo), 10 g; ferric ammonium citrate, 0.5 g; agar, 13 g; and distilled water, 1000 ml. Gluconate-iron agar was prepared as slants and inoculated by the streak and stab technique; however, since the pseudomonads do not grow to any extent in the stab, it is only necessary to streak the slanted surface. Positive

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hydrogen sulfide reactions on this medium by pseudomonads range from slight discoloration to complete blackening in the surface growth. Six enteric organisms, *Escherichia freundii, Salmonella schottmuelleri, S. enteritidis, S. typhosa, Proteus vulgaris,* and *P. mirabilis,* gave positive hydrogen sulfide reactions on triple sugar iron agar but were negative on gluconate-iron agar. This medium was also used to evaluate chromogenesis.

Triple sugar iron agar (TSI agar, BBL) was used for hydrogen sulfide instead of Kligler's iron agar used by Rozansky, *et al.* (1950). Any browning to blackening of the surface growth was considered a positive reaction. Trypticase soy broth (BBL) was used to observe pellicle formation and for the broth cytochrome oxidase test of Gaby and Hadley (1957). The cytochrome oxidase test was considered positive if some degree of blue appeared within 30 seconds. Trypticase soy agar (BBL) plates were used to observe colonial morphology and for the plate cytochrome oxidase test. This test was considered positive if the colonies turned blue within 30 seconds. Trypticase soy agar slants were inoculated to detect chromogenesis and for the paper oxidase test of Kovacs (1956). This test was considered positive if the paper and the culture material turned dark purple within 30 seconds. Sabouraud maltose agar (Difco) slants were used for chromogenesis following the recommendations of Martineau and Forget (1958).

D-arabinose broth was used to determine acid production from this carbohydrate. Simon (1956) showed that sugar fermentations of *Pseudomonas* spp are more readily detected in carbohydrate media containing a minimum of organic nitrogenous materials and further stated that of 13 species of *Pseudomonas* studied by her only *P. aeruginosa* fermented D-arabinose. Preliminary trials indicated that broth consisting of 1 per cent D-arabinose and 0.1 per cent trypticase with brom cresol purple as the pH indicator could readily be used to detect acid production by *P. aeruginosa.*

Nitrate agar (Difco), nutrient gelatin (Difco), glucose broth (Difco), and urease test medium (BBL) were used for their respective functions. All inoculated media, with uninoculated controls, were incubated at 37 C for such time as was considered appropriate for the specific determinations.

Results

On the basis of a combination of characteristics, namely chromogenesis, hydrogen sulfide production, cytochrome oxidase test in broth, and nitrate reduction, eighteen of the 23 cultures were considered to be species of *Pseudomonas.* Of the remaining five cultures two were designated *A. faecalis,* one was considered to be *Bacterium anitratum* (Schaub and Hauber, 1948), and two remain unidentified.

All of the 18 pseudomonads gave positive cytochrome oxidase tests in broth and on agar, were oxidase positive by Kovacs test, reduced nitrate, liquefied gelatin within six days, and were urease negative. All but one showed some degree of pellicle formation. Table 1 shows the number of cultures positive for the remaining characteristics for the first five days and on the twelfth day of incubation. Table 2 shows the day-by-day positive cultures for pigmentation and hydrogen sulfide formation on the various media used. One of the twelve cultures which

TABLE 1

Number of Strains of P. Aeruginosa Positive for Four Characteristics

Days After Inoculation	Pigment	H_2S	Acid From Glucose	Acid From Arabinose
1	16	17	3	2
2	16	18	6	11
3	17	..	8	14
4	18	..	12	15
5	..	..	15	17
12	..	..	15	18

TABLE 2

Variation of Pigment and H_2S Production by P. Aeruginosa in the Media Utilized

Days After Inoculation	PIGMENT				H_2S	
	TSAS*	TSAP	SMA	GI	TSI	GI
1	9**	10	16	7	5	17
2	9	..	16	10	8	18
3	9	..	16	12	11	..
4	9	..	17	12	11	..
5	10	..	17	12	11	..

* TSAS, tryticase soy agar slant; TSAP trypticase soy agar plate; SMA, Sabouraud maltose agar; GI, gluconate iron agar; TSI, triple sugar iron agar.
** No. of strains positive.

produced pigment on gluconate-iron agar did not produce pigment on Sabouraud maltose agar within 10 days of observation. For the same characteristics none of the non-pseudomonads gave positive cytochrome oxidase tests, produced pigment, hydrogen sulfide, or acid from arabinose, two were oxidase positive, two reduced nitrates, two liquefied gelatin, five were urease negative, two formed pellicles, and one produced acid from glucose.

Twelve of the pseudomonads produced flat, effuse, granular, spreading colonies on trypticase soy agar. These were considered to be the (a) and (c) type colonies of Gaby and Free (1953). Six produced raised, convex, finely granular colonies with entire edges. These were considered to be the (b) type colonies of Gaby and Free. All of the non-pseudomonads produced the smooth type colony.

Discussion

Pseudomonas aeruginosa has long been associated with human infections as a secondary invader. In recent years with the advent of prolonged therapy with wide spectrum antibotics its role as a primary cause of disease has increased in significance (Forkner, *et al.*, 1958), but the identification of this organism in the clinical laboratory is still a technical problem. As Gaby and Free (1953, 1958) have pointed out, the typical, pigmented strains are readily identified, but the achromogenic strains which produce raised, confined, entire colonies are easily confused with Alkaligenes and with other organims producing similar biochemical reactions on triple sugar iron agar (Schaub and Foley, 1952).

The hydrogen sulfide reaction of *Pseudomonas aeruginosa*, apparently long ignored as a characteristic for identification, appears to be no more variable, in fact may be less variable, than some of the other characteristics commonly used to identify this species. As pointed out by

Rozansky, *et al.* (1950), the blackening appears initially in the surface growth in contrast to the diffuse blackening below the slanted surface and the colorless surface growth that results with more typical hydrogen sulfide-positive bacteria. A comparison of the appearance of hydrogen sulfide reactions of pseudomonads and enteric organisms on a medium such as Kligler's iron agar or triple sugar iron agar would seem to indicate that the reaction of the former is an aerobic process, whereas that of the latter is anaerobic. When the pseudomonad cultures are held for several days, the blackening slowly diffuses into the medium, frequently forming a line marking the extent of diffusion of pyocyanin. When the hydrogen sulfide reaction is very marked in the first 24 hours, pyocyanin is frequently not observed; however, after longer incubation diffusion of pyocyanin becomes evident. This would seem to indicate that in the presence of the reducing conditions pyocyanin is maintained in the reduced state, but as the hydrogen sulfide producing activity of the culture lessen the pyocyanin becomes oxidized.

Acid production from carbohydrates by pseudomonads varies with the concentration of organic nitrogen and the method of determination (Salvin and Lewis, 1946; Liu, 1952; Ringen and Drake, 1952; Simon, 1956); pigmentation with the type of peptone or carbohydrate (Ringen and Drake, 1952; Martineau and Forget, 1958). In this study acid production was more readily detected from arabinose in dilute trypticase broth than from glucose in peptone-extract broth. The number of cultures producing pigment was 10, 12, and 17 on the three media used for this characteristic.

The broth cytochrome oxidase test of Gaby and Hadley (1957) readily separates pseudomonads from other organisms giving similar reactions on triple sugar iron agar; however, in this study the delayed positive reactions, considered by Gaby to be characteristic of Alkaligenes (Gaby and Hadley, 1957; Gaby and Free, 1958), was not considered valid since the same reaction was obtained with uninoculated trypticase soy broth. As pointed out by Gaby and Free (1958) the oxidase test of Kovacs (1956) is too sensitive; however, in this study the agar plate test of Gaby and Hadley (1957) also seemed overly sensitive since the two strains of Alkaligenes were positive in 30 seconds by this procedure as well as by the Kovacs test.

The procedure used by Simon (1956) to detect acid production from carbohydrates seems impractical for routine use. The simple broth used in this study for the detection of acid production from D-arabinose is easily prepared and can be readily used in any laboratory.

The establishment of constant characteristics for a particular species of bacteria is difficult when so many possible formulations for media for the detection of these characteristics exist. But that a biochemical characteristic as commonly determined as hydrogen sulfide production for as old and as common a bacterium as *Pseudomonas aeruginosa* should go unemphasized for so long a time seems unusual. There is no doubt that *P. aeruginosa* produces hydrogen sulfide in the same sense that the *Enterobacteriaceae* do. Even though strain production varies qualitatively with the media used and quantitatively with any individual medium, this characteristic of *P. aeruginosa* is no more variable than chromogenesis,

acid production from glucose, or colonial morphology, three characteristics on which identification of this species is frequently based (Gaby and Free, 1953). Haynes (1951) indirectly summed up these variations when he stated, "It is apparent that the certain identification of *P. aeruginosa* requires the use of media and conditions specifically designed and proved most suitable to ellicit characteristic responses. Failure to do so is bound to result in misnaming of many isolates."

Commercial production of a medium, such as gluconate-iron agar, to aid in the identification of a single species of bacterium is undoubtedly impractical; however, increased numbers of atypical strains of *P. aeruginosa* could be differentiated from grossly similar appearing organisms by hydrogen sulfide production if media such as Kligler's iron agar or triple sugar iron agar were observed beyond the usual limits of 24 hours.

As a result of this study it is believed that the Pseudomonas-Alkaligenes-Mimeae *(B. anitratum)* group can be readily differentiated in the following manner: Organisms which produce alkaline slant-alkaline butt reactions on Kligler's iron agar or on triple sugar iron agar are transferred to gluconate-iron agar, Sabouraud maltose agar, trypticase soy broth, nitrate agar, 10 per cent lactose agar (Schaub and Hauber, 1948), glucose broth, 1 per cent D-arabinose-0.1 per cent trypticase broth. After the proper periods of incubation the organisms are differentiated by the reactions shown in table 3.

TABLE 3

Biochemical Differentiation of Pseudomonas-Alkaligenes-Mimeae Group

	H_2S	Pigment	Cyto Ox <30 Sec.	NO_3 red	Acid From 10% Lactose	Acid From Glucose	Acid From D-arabinose
P. aeruginosa	+	+	+	+	—	+ slow	+
Alkalingenes spp.	—	—	—	+	—	—	—
B. antitratum (Mimea)	—	—	—	—	+	+ rapid	+ —

Acknowledgment

The author wishes to thank Mrs. Jenetta Kirkwood and Mrs. Laura Belle Richardson for their assistance in the preparation of media.

Summary

A comparison is made of hydrogen sulfide production by *Pseudomonas aeruginosa* with other characteristics used to identify this organism. Of 23 cultures giving identical reactions on triple sugar iron agar 18 were identified as *Pseudomonas.* Of these eighteen cultures 18 produced hydrogen sulfide on gluconate-iron agar, 8 on triple sugar iron agar, within 48 hours; 17 produced greenish to bluish-green pigment on Sabouraud maltose agar within 3 days, 12 on gluconate-iron agar in 3 days, 10 on trypticase soy agar in 5 days; 15 produced acid from glucose, 17 acid from D-arabinose in 5 days (18 in twelve days); 17 of the cultures showed some degree of pellicle formation in trypticase soy broth; 18 gave positive cytochrome oxidase (Gaby) and oxidase (Kovacs) tests,

reduced nitrates, liquefied gelatin and were urease negative; 12 of the cultures formed rough type colonies, 6 smooth colonies.

Of the 5 non-pseudomonads two were identified as *Alkaligenes faecalis*, one as *Bacterium anitratum* and two remained unidentified.

A procedure is given for the differentiation of the Pseudomonas-Alkaligenes-Mimeae group.

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COMPARISON OF ALKALINE PHOSPHATASE METHODS†

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The osteoblastic cells of the bone are believed to be the site of origin of a phosphatase whose greatest activity is at about pH 9.5. This phosphatase, commonly termed "alkaline" phosphatase, is normally excreted by the liver into the bile. In cases of obstructive jaundice the phosphatase is regurgitated into the blood stream along with the bile; therefore, alkaline phosphatase determinations are considered a means of differentiating obstructive from infectious jaundice as well as being a measure of liver function and bone activity. For these reasons the alkaline phosphatase determination is frequently requested and we felt the need of investigating a simpler procedure rather than continuing the use of one of the two commonly used methods, the Bodansky or the King and Armstrong. In recent years two other methods have come into use in the laboratory, the Bessey-Lowry and "Phosphatabs". Both methods are far simpler and less time consuming than either the Bodansky or the King and Armstrong procedures. The purpose of this paper is to show a comparison between the four methods and to determine the reproducibility of the results.

PROCEDURES

I. Bodansky

The Bodansky method for alkaline phosphatases is a variation of the original Kay method. The procedure used in this series was the Barringer and Woodward modification of the Bodansky method using a B-glycerophosphate substrate with a pH of 8.6. The substrate was purchased in dried form from a commercial source.* One ml. of serum and 9 ml. of substrate were incubated for 1 hour at 37° C. Inactivation of the enzyme and precipitation of the protein were accomplished by addition of trichoroacetic acid. Phosphorus determinations by the Fiske and SubbaRow method were run on the resulting filtrates from both incubated and non-incubated samples. The tests were read on a Coleman Universal Spectrophotometer at 660 mu. Normal values were 1.5-4.0 units/100 ml.[2, 6]

II. King and Armstrong

This method, also a variation of the original Kay method, utilizes the liberation of phenol from disodium monophenyl phosphate. The substrate used had a pH of 9.3 and was purchased commercially in dried form.* Five-tenths ml. serum and 10 ml. substrate were incubated at 37° C. for 30 minutes. Precipitation of the protein and inactivation of the enzyme were accomplished by using a dilute phenol reagent consisting of a mixture of phosphomolybdic and phosphotungstic acids. The stock phenol reagent was also purchased through a commercial source.* The color was developed on both incubated and non-incubated filtrates by the addition of sodium carbonate, and the results were read on a Coleman Universal Spectrophotometer at 660 mu. Normal values were 4-13 units/100 ml.[4, 6]

† Read by title at 27th Annual Convention of A.S.M.T., Phoenix, Arizona, June 1959.
*Harleco Reagents.

III. Bessey-Lowry

The Bessey-Lowry method was first reported in this country by Bessey, et. al. in 1946.[1] This method has not been used generally because of the difficulty in obtaining the p-nitrophenol phosphate substrate in pure and stable form. We purchased both the substrate and buffer from a chemical company.** The substrate was dispensed in 0.5 ml. quantities into screw capped tubes which were kept in a deep freeze until use. Just prior to testing, the tubes were thawed and 0.5 ml. buffer (ph 10.5) was added. The resultant buffered substrate was warmed to 37° C. and 0.1 ml. serum was added. This mixture was incubated for 30 minutes at 37° C. which resulted in the quantitative liberation of p-nitrophenol, a salt, which is colorless in an acid solution and yellow in an alkaline solution. Inactivation of the enzyme and color development were accomplished by adding 0.02N sodium hydroxide. Results were read immediately at 470 mu. on the Coleman Universal Spectrophotometer against a substrate blank. Correction for serum color was made by the addition of 2 drops of concentrated hydrochloric acid and re-reading. Normal values were 0.8-2.3 B.L. units/100 ml.[1, 5]

IV. "Phosphatabs"

"Phosphatabs" were run according to the directions given by the supplier,* using the 12 minute incubation at 37° C. Here, the substrate was a stabilized sodium phenolphthalein phosphate in a buffer that gave a pH of 9.8 with serum. In the reaction, the phosphate was split and the free phenolphthalein was used to measure the enzyme activity. Since there is practically no blank color and, of course, no phenolphthalein in normal serum, the color developed was a measure of enzyme activity.

RESULTS

Serum from twenty patients was run in duplicate by each of the four methods mentioned above. We included three normals along with what we considered a range of values usually found in the laboratory. The serum was added to the warmed substrate (37° C.) at timed intervals and inactivation was carried out at the same timed intervals. New calibration curves were run for each procedure just prior to the start of this series of analyses. The results, shown in Tables I and II, include the duplicate values by each of the four methods and the clinical diagnoses of our patients.

DISCUSSION

From observation of this series we feel that the correlation between the Bessey-Lowry, King and Armstrong and "Phosphatabs" methods was very good; however, the Bodansky values seemed low. Considering that the upper limits of normal on the Bodansky method are accepted as 4 units, we found that many of our results would be considered normal by this method and yet were elevated by the other methods. The elevated values seemed to correspond with the clinical diagnoses. One of our controls was at the upper limit of normal by all methods except that of Bodansky. There was no clinical evidence of any disease in this case and repeat determinations a month later gave the same results

*Warner-Chilcott
**Sigma Chemical Company

TABLE I

Case	Bessey-Lowry 0.8–2.3 Units	King-Armstrong 4–13 Units	Bodansky 1.5–4 Units	Phosphatabs	Clinical Diagnosis
No. 1	0.8 units 0.8 units	4 units 6 units	0.9 units 0.7 units	Normal Normal	Control
No. 2	0.8 units 0.8 units	6 units 6 units	1.0 units 1.1 units	Normal Normal	Control
No. 3	1.9 units 1.9 units	14 units 12 units	1.7 units 1.9 units	Between Normal and Borderline	Control
No. 4	3.4 units 3.4 units	20 units 21 units	2.6 units 2.5 units	Borderline Borderline	Biliary Obstruction
No. 5	4.1 units 4.1 units	23.5 units 23 units	3.8 units 3.4 units	Low Pathological Low Pathological	Biliary Obstruction
No. 6	2.4 units 2.4 units	22 units 20 units	2.4 units 2.5 units	Borderline Borderline	Biliary Obstruction
No. 7	5.7 units 5.6 units	41 units 42 units	5.2 units 5.6 units	Low Pathological Low Pathological	Biliary Obstruction
No. 8	7.9 units 7.9 units	59 units 57 units	9.3 units 9.4 units	High Pathological High Pathological	Biliary Obstruction with a large stone in the common bile duct.
No. 9	32.6 units 33.0 units	234 units 204 units	41.8 units 42.6 units	High Pathological High Pathological	Biliary Obstruction due to compression of the bile duct by tumor.
No. 10	5.9 units 5.9 units	53 units 46 units	5.6 units 5.8 units	High-Low Pathological High-Low Pathological	Carcinoma-head of the pancreas with extension to the bile duct.

TABLE II

Case	Bessey-Lowry 0.8–2.3 Units	King-Armstrong 4–13 Units	Bodansky 1.5–4 Units	Phosphatabs	Clinical Diagnosis
No. 11	12.9 units 11.8 units	63 units 60 units	10.3 units 10.3 units	High Pathological High Pathological	Carcinoma-head of the pancreas with duodenal obstruction.
No. 12	5.0 units 4.9 units	27 units 27 units	4.1 units 4.2 units	Low Pathological Low Pathological	Choledocholithiasis
No. 13	3.0 units 3.4 units	15 units 16 units	2.2 units 2.2 units	Borderline Borderline	Choledocholithiasis
No. 14	2.3 units 2.4 units	13 units 13 units	1.6 units 1.6 units	Borderline Borderline	Carcinoma of the gall bladder.
No. 15	5.0 units 5.1 units	35 units 33 units	3.6 units 3.6 units	Low Pathological Low Pathological	Hepatitis of long standing.
No. 16	4.1 units 4.1 units	23 units 23 units	3.2 units 3.3 units	Low Pathological Low Pathological	Mild hepatitis.
No. 17	4.2 units 4.1 units	27 units 28 units	4.7 units 4.7 units	Low Pathological Low Pathological	Cirrhosis
No. 18	6.3 units 6.3 units	48 units 42 units	5.9 units 5.9 units	High Pathological High Pathological	Cirrhosis
No. 19	6.3 units 6.3 units	46 units 43 units	5.9 units 6.2 units	High Pathological High Pathological	Advanced osteoarthritic changes in the bone.
No. 20	2.4 units 2.3 units	14 units 17 units	1.9 units 1.9 units	Borderline Borderline	Carcinoma with bone metastases.

From the charts one can easily see that "Phosphatabs" are quite accurate. Although they are not quantitative, they definitely could have a place in small hospitals, clinics and doctors' offices.

We feel that the Bessey-Lowry method should be given more attention. We became interested in this procedure because it was a simple one-step test, took less time, and used smaller quantities of serum. Since the same substrate could be used for both acid and alkaline determinations, the procedure was less expensive. Being a one-step procedure, the chance of error was lessened and from the above charts one can see that the results were reproducible.

Another advantage of the Bessey-Lowry and "Phosphatabs" methods is that the substrate, if properly stored, does not deteriorate and therefore have to be renewed weekly. This is especially advantageous in the small hospital laboratory where a phosphatase determination may rarely be requested.

SUMMARY

A series of twenty analyses have been presented to show the correlation between the Bessey-Lowry, Bodansky, King and Armstrong and "Phosphatabs" methods for alkaline phosphatases. All tests were run in duplicate by each of the four methods and the results showed good reproducibility. The Bessey-Lowry, King and Armstrong and "Phosphatabs" methods showed good correlation between themselves and the results agreed with the clinical diagnoses. The Bodansky method gave comparably lower results. From this set of analyses we felt that the simpler procedures, such as Bessey-Lowry and "Phosphatabs", should be given more attention by the routine clinical laboratory.

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FURTHER STUDIES ON THE PHENOTYPE Jk(a—b—)*

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The finding of Anti-Jk^a in 1951 by Allen and his associates,[1] followed by the discovery of Anti-Jk^b some two years later by Plaut et. al.,[4] established a blood group system which was new and independent of all other known systems.

All bloods tested with these two antisera proved to be positive with one or the other or positive with both. Statistically and by actual test, it appeared that the Kidd System was a straight forward two allele system. This presented then, three possible phenotypes as determined by tests on English people.

TABLE No. 1

Phenotype	Occurrence
Jk (a+b—)	25%
Jk (a+b+)	50%
Jk (a—b+)	25%

Tests of other ethnic groups revealed some differences in the frequency percentages, but the three basic phenotypes still held.

In August of 1958, a blood of the phenotype Jk(a—b—) was found for the first time.

The blood of Mrs. S. was sent to the Knickerbocker Foundation, New York, by Drs. Pinkerton and Mermod of the Blood Bank of Hawaii in Honolulu. Mrs. S., a Filipino of Spanish and Chinese ancestry, had become jaundiced five days after transfusion of four pints of blood. Her serum was found to contain an antibody reactive with all cells tested except her own.

Mrs. S. had two children, neither of whom had suffered from hemolytic disease. Her parents were not related as far as was known, and unfortunately, no sibs were available for testing.

The first clue to the identity of this antibody came, when it was decided to test her cells with all the various antisera available. Her cells failed to react with Anti-Jk^a serum and Anti-Jk^b serum. A number of other examples of these antibodies were then tried, and her cells again failed to agglutinate. In all, nine different Anti-Jk^a and eight different Anti-Jk^b sera were tried, all of which gave expected results with known control cells. Mrs. S.'s red cells were not unusual in any other way.

These results, of course, presented something new in the Kidd system, and samples were sent to Drs. Race and Sanger at the Lister Institute in England. They were able to very quickly confirm the fact that Mrs. S. was indeed Jk(a—b—).

With this knowledge as a guide, further tests of the antibody in her serum suggested that she had some separable Anti-Jk^b plus an inseparable Anti-Jk^{a}Jk^b. Details of this work are reported in a paper by Pinkerton, Mermod, Liles, Jack and Noades.[3]

Returning to the cells of Mrs. S., we now find, for the first time in

* Read before the 27th Annual Convention of ASMT, Phoenix, Arizona, June 1959.

the Kidd system, a phenomenon encountered earlier in most other systems—namely, a minus-minus phenotype. Two familiar examples are found in the Lewis and Duffy systems. In the Lewis system 6% of white and 22% of negro bloods present this phenotype.[5] In the Duffy system, although no white blood has shown this phenotype, 68% of negro bloods have.[7]

The simplest genetical explanation for the Jk(a—b—) phenotype would be the postulation of a third allele at the Kidd locus, for example, Jk, for which Mrs. S. is then homozygous. Should the corresponding antibody be found, it would establish the existence of the third allele, which would then be called Jk^c.

Unfortunately, no family study was possible in the case of Mrs. S.

A survey of white and negro donors was undertaken in an attempt to find another blood of the Jk(a—b—) phenotype.

A total of 505 bloods were tested with the serum of Mrs. S. Of these, 326 were white and 179 negro, as determined on the basis of skin color only. All bloods tested gave strong positive reactions with her serum.

In 1953, Dr. Rosenfield and his associates made a study of New York Chinese and found that of 103 bloods tested, only 52% gave positive results with Anti-Jk^a serum.[6] This is the lowest incidence reported in some ten ethnic groups tested. All other ethnic groups showed a positive incidence of Jk^a ranging from 62% in British Columbian Indians to 100% in Eskimos and the Sea Dyaks of Borneo.[2]

As Mrs. S. herself contained some Chinese ancestry, it was felt that Chinese blood would present a likely source of another Jk(a—b—) phenotype.

With the cooperation of Dr. Leon Sussman of the Beth Israel Hospital in New York City, we were able to test some 26 samples of New York Chinese. Again, in every case, the serum of Mrs. S. gave positive results. Further check of these bloods with Anti-Jk^a and Anti-Jk^b typing sera showed all to be positive for Jk^a, Jk^b, or both.

Although 26 bloods is not a large sampling of an ethnic group, when it is added to the 103 specimens tested by Dr. Rosenfield and his group, the number becomes significant. Certainly, even if the Jk(a—b—) phenotype does exist in Chinese blood, it represents a very small percentage.

Other such ethnic groups remain to be tested. Should this research fail to disclose another Jk(a—b—) phenotype, another theory may have to be adopted to explain this one. Other possibilities besides the third allele, would be the effect of a modifying gene or gene deletion. Certainly further work is indicated to supply the answer to the Jk(a—b—) phenotype.

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MEDICAL TECHNOLOGISTS WANTED

Medical Technologist: Registered ASCP or eligible. 316 bed modern general hospital. 3 full time pathologists. Approved school of Medical Technology. No night calls. 37½ hour week. Starting salary based on experience and education. Living accommodations on premises when available may be arranged for female technologist if desired. Fine suburban community 20 miles from New York City. Easy access to shore resorts. Apply Personnel Office, Overlook Hospital, Summit, New Jersey.

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Wanted: Medical Technologists, (ASCP) or eligible. Salary starting at $3640-$4290 and $3770-$4940 respectively and experienced medical technologist qualified for laboratory and teaching supervisor starting at $5460-$6370 depending on qualifications for 182 bed general hospital expanding modern laboratory and approved school in town of 89,000 located 45 minutes from N. Y. C. Contact: Pathologist, St. Joseph's Hospital, Stamford, Connecticut.

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(Continued on Page 148)

A REPORT OF QUANTITATIVE SEROTONIN DETERMINATIONS IN METASTATIC CARCINOID*

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We have had the opportunity of studying a case of metastatic carcinoid tumor of the small intestine and performing qualitative and quantitative tests for serotonin on this case. Here we would like to present the easily-adaptable method of preserving the positive specimen for teaching purposes and later use as a positive control.

History

Serotonin is derived from the essential amino acid, tryptophane, and the greater part of serotonin formed in the body is excreted as 5-hydroxyindoleacetic (5HIAA). Tryptophane undergoes oxidative decarboxylation to form serotonin or 5-hydroxytryptamine which is then transformed into 5-HIAA by the enzyme amine oxidase. Ersparmer and his associates identified 5-hydroxytryptamine in 1952.[2] Serotonin is apparently elaborated by the cells of the enterochromaffin system, the precursor being tryptophane. Biorck discovered that some carcinoid tumors secreted serotonin and observed an associated syndrome consisting of such changes as flushing, cyanosis, diarrhea and skin changes.[1] These tumors arise from the enterochromaffin cells of the intestinal mucosa. All of these tumors are not benign but may be malignant and metastasize.

Case

On July 31, 1958, a 49-year-old white male entered the hospital for a hemorrhoidectomy, and in August a biopsy was made of the pelvic peritoneum and omental lymph nodes, with a final diagnosis of metastatic carcinoid. Following surgery, he received a course of radiation therapy via various abdominal portals. Diarrhea persisted with severe cramps through September. His hemoglobin of 10.4 gm. % and hematocrit of 32 vols. % remained unchanged after two units of blood and his temperature was never below 98° F nor exceeded 100° F. He grew weaker and continued to lose weight while edema developed in his feet and lower legs. The diarrhea apparently subsided, but there was persistent abdominal distention with clinical evidence of peritoneal fluid. The patient expired on November 20, 1958.

Reagents

1-nitroso-2-napthol. (A.R.) One-tenth per cent in 95 percent ethanol.

Nitrous acid reagent. (A.R.) Two-tenths of a milliliter of 2.5 per cent sodium nitrite (A.R.) added to 5 ml. of 2 N sulfuric acid (A.R.). Prepare fresh the day of use.

Sodium chloride. (A.R.) Two grams per test.

Ether (anesthetic grade).

Ethyl acetate. (A.R.)

Ethylene chloride. (A.R.)

5-hydroxyindoleacetic acid standard. (A.R.)

The stock standard solution was made by weighing 50 mg. of the acid standard and dissolving in 500 ml. of distilled water. The pH was

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kept at approximately 3 by occasionally adding a few drops of glacial acetic acid. One ml. equals 100 mg. By diluting 2.5, 5.0, 10., 12.5, 15.0, 17.5, 20.0, and 22.5 ml. of stock 5-HIAA standard to 25 ml. with distilled water, we obtained dilutions from 10 to 90 mg. of 5-HIAA. The standards were measured at 650 mu against a reagent blank set at 100 per cent transmittance. A curve was plotted on semilogarithmic paper giving results in mg.

Methods

The qualitative test performed was first developed by Sjoerdsma and co-workers and reported in 1955.[3]

To 0.2 ml. of urine, add 0.8 ml. of distilled water and mix. Add 0.5 ml. of 1-nitroso-2-napthol reagent and 0.5 ml. of nitrous acid reagent, mix and let stand ten minutes. Add 5 ml. of ethylene chloride, mix and centrifuge. A purple color in the upper layer indicates a positive test.

The quantitative test used was that described by Pierce.[1]

In a large 50 ml. centrifuge tube, place 5 ml. of a 24-hour urine specimen, to which had been added 25 ml. of glacial acetic acid prior to collection; 2 gm. of NaCl; and 25 ml. of ether. Stopper, mix well for at least one minute and centrifuge for one minute at 2000 r.p.m. Place 20 ml. of the ether layer in a 50 ml. Erlenmeyer flask and evaporate to dryness in a 56° C water bath. To the residue, add 4 ml. of distilled water and let stand for five minutes. In a large tube place 2 ml. of the water extract; 1 ml. of 1-nitroso-2-napthol reagent, and 1 ml. of nitrous acid reagent. Mix well and place in a 56° C water bath for five minutes. Remove and place immediately in ice water. Add 10 ml. of ethyl acetate, mix and let stand 30 minutes at room temperature. Discard the top ethyl acetate layer and read the bottom layer at 650 mu on any suitable spectrophotometer. Obtain mg. value for simultaneously-determined standard curve.

$$\frac{\text{24 hour volume X mg.}}{1000} = \text{mg./24 hours}$$

Results

A voided urine specimen was collected two weeks after surgery on the above patient and a 5-HIAA was performed and proved to be positive by the qualitative method. A 24-hour specimen was then collected for quantitative study and found to be 141 mg./24 hours, whereas, the normal is 2-8 mg./24 hours. After the patient expired, fluid was obtained from the peritoneal cavity and urinary bladder. Only the fluid from the urinary bladder proved to contain 5-HIAA.

Preservation

In order to preserve the original specimen, it was collected in a container with 25 ml. of glacial acetic acid. The specimen, after collection, was filtered through Whatman #40 filter paper. The sample taken at necropsy was neither filtered nor acidified. Each of the samples were placed in empty 600 ml. plastic transfer bags from the Blood Bank and frozen at approximately —5° C.

Discussion

Once every two weeks the bags of urine were thawed in a 37° C water bath and 10 ml. portions were extracted slowly from the end of

the tubing with a sterile 25 gauge needle. Quantitative determinations were performed on the samples and bags refrozen. There was apparently no bacterial contamination during the length of time of the experiment, nor was the bag damaged because of the repeated freezing and thawing or by repeated puncture with the small-gauge needle. After four months, it was noted that the results were the same as the original values found and the 5-HIAA had not decreased. During the experiment the artificial standards remained constant for only one month at a pH of 3 and refrigeration. In freezing these bags, the top freezing compartment of an ordinary laboratory refrigerator was used and the temperature was never below —5° C. Also it was noted that there was no noticeable difference in stability as a result of either preliminary filtering or preliminary acidification.

A Beckman model B spectrophotometer was used during this experiment, but any similar spectrophotometer, or also filter photometer could be adapted to this procedure. This test, though seldom required, would be an interesting addition to any laboratory's roster of determinations. The seeming relative stability of a positive urine sample in the frozen state would aid teaching of technics to student technologists and/or new personnel by having actual samples to work with rather than artificial or pure standards.

Both the qualitative and quantitative tests are simple to perform. The qualitative requires only fifteen to twenty minutes; the quantitative requires about an hour for the entire procedure. Preservation is neither expensive nor difficult. Storing is advantageous for later use of the specimens for positive controls and in teaching new students and technologists entering the laboratory.

Summary

Serotonin is derived from the essential amino acid tryptophane and the greater part formed in the body is excreted as 5-HIAA. In July 1958, a patient entered the hospital for a hemorrhoidectomy. Later a diagnosis of malignant carcinoid, primary in the ileum, with wide-spread abdominal metastases, was made. Urine specimens, positive for 5-HIAA, were collected during illness and at necropsy. The specimens were preserved in plastic bags and frozen for later use as positive controls and for teaching purposes.

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